Journal of Plant Protection and Pathology

Journal homepage & Available online at: www.jppp.journals.ekb.eg

Toxicity and Nutritional Disruptions Induced by *Aspergillus melleus* Alkaline Protease in Insect Larvae

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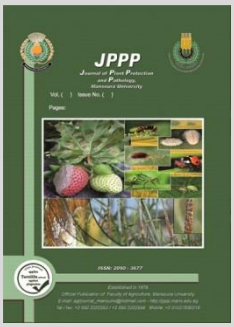

ABSTRACT

This study investigates the insecticidal potential of *Aspergillus melleus* protease on *Galleria mellonella* larvae, revealing significant biological effects, including increased mortality, developmental delays, adverse impacts on nutritional indices, and histopathological changes. Fungal spores were isolated from soil in El-Khatatba and cultured to produce protease enzyme, with optimal production occurring at pH 8, 35°C, and 72 hours of incubation. A dose-dependent increase in larval and pupal mortality was observed, with LC_{50} and LC_{90} values determined as 1.9 × 10^6 and 2.5 × 10^8 spores/mL, respectively. Spore concentrations significantly delayed the larval and pupal development and induced morphological deformities in *G. mellonella*. Histopathological examination revealed damage to the midgut epithelial layer of the larvae, contributing to impaired nutrient absorption. Nutritional indices revealed a marked decrease in relative growth and consumption rates, as well as reduced conversion efficiency and increased feeding deterrence. These findings highlight the potential of *A. melleus* protease as a bio-insecticide, with *G. mellonella* serving as a model insect for evaluating its effectiveness.

Keywords: Entomopathogenic fungi; Protease activity; Insect nutritional indices; Histopathology; Biological Control.

INTRODUCTION

Biological control is increasingly recognized as an essential strategy for maintaining environmental health and protecting human populations from the risks associated with synthetic chemical pesticides (Baker *et al.* 2020). Microorganisms, in particular, have emerged as key biological control agents due to their ability to produce compounds with pesticidal properties, including enzymes like proteases that disrupt critical physiological functions in insects (Bonaterra *et al.*, 2022). One such microorganism, *Aspergillus melleus*, produces alkaline proteases that have demonstrated potential as bio-insecticides by targeting essential proteins and tissues within the insect host (Harrison and Bonning, 2010).

The greater wax moth, *Galleria mellonella* L (Lepidoptera: Pyralidae), serves as a model organism for evaluating microbial virulence due to its well-documented immune responses, ease of rearing, and physiological similarities to economically important insect pests (Tsai *et al.*, 2016; Jorjão *et al.*, 2018). Its use in studies involving entomopathogenic fungi is well-established, making it an ideal model for assessing the insecticidal potential of fungal proteases (Namara *et al.*, 2017; Asai *et al.*, 2023). In particular, the midgut of *G. mellonella*, which plays a critical role in digestion and defense against pathogens, is a key site for investigating the histopathological impacts of protease exposure (Keppanan *et al.*, 2017).

Proteases secreted by fungi like *A. melleus* are of particular interest in biological control because of their dual role in toxicology and nutrition disruption. These enzymes not only contribute to the pathogenicity of microbial pathogens by degrading critical proteins in the host but also interfere with the host's ability to process and absorb nutrients, leading to growth inhibition and mortality (Harrison and Bonning, 2010; Fang *et al.*, 2009). The degradation of the midgut's protective peritrophic membrane, which shields the epithelium from pathogens and regulates digestive processes, is a critical target for protease activity (Whiten *et al.*, 2018). Damage to this barrier can result in impaired nutrient absorption, delayed development, and eventual death of the larvae (Zeng *et al.*, 2022; Zhang and Edgar, 2022).

The midgut, hemocoel, and cuticle of insects are potential sites for protease-induced damage, and the histopathological examination of these tissues is essential for understanding the full range of effects caused by fungal proteases (Semenova *et al.*, 2020). In addition to histological damage, these enzymes disrupt key nutritional indices, including the relative growth and consumption rates of insect larvae, as well as the efficiency with which they convert ingested food into biomass (Hussain *et al.*, 2009). Thus, fungal proteases not only act as direct toxins but also as disruptors of insect physiology.

Given the critical role of proteases in insect pathology, the aim of this study is to investigate the insecticidal, histopathological, and nutritional effects of *A. melleus* alkaline protease on *G. mellonella* larvae. By exploring the dual roles of toxicity and nutritional disruption, alongside tissue damage, this study provides a comprehensive evaluation of *A. melleus* protease as a potential bio-insecticide.

MATERIALS AND METHODS

Organism and Inoculum Preparation

The fungal strain *A. melleus* was isolated from the soil of planted fields in El-Khatatba, Alexandria Governorate,

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DOI: 10.21608/jppp.2024.323481.1267

Egypt. Isolation was conducted using the serial dilution plate method (Waksman, 1922), with dilutions ranging from 10^{-1} to 10^{-4}, plated onto Potato Dextrose Agar (PDA) medium. Fungal colonies exhibiting the highest protease activity were selected based on clear zones around the colonies. These cultures were purified by routine sub-culturing and stored at 4°C for further use. The fungal strain was genetically identified to confirm its taxonomic classification.

Preparation of Fungal Spore Suspensions

Spores of *A. melleus* were cultured on PDA medium in Petri dishes for 10 days at 28°C. Once grown, the spores were harvested by scraping the surface of the medium and suspending them in distilled water containing 0.1% Tween 80. The conidial concentrations were then adjusted using a hemocytometer to 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/mL (Lacey, 2012). These spore suspensions were used as the stock for insect treatments (Abdel-Rahman & Reda, 2019).

Screening of *A. melleus* Protease Production

To evaluate the production of protease by *A. melleus*, the fungus was cultured on skimmed milk agar plates at 30°C for 5 days. Protease activity was assessed by measuring the diameter of the clear zone formed due to the hydrolysis of skim milk, which indicates proteolytic enzyme activity.

Effect of Incubation Period, pH, and Temperature on Proteolytic Activity

The proteolytic activity of *A. melleus* was assessed under various incubation periods, pH levels, and temperatures. A 50 mL casein yeast extract broth medium was prepared and inoculated with two 10 mm fungal mycelial discs. The incubation periods tested ranged from 48 hours to 8 days at 28°C. Protease activity was evaluated at different temperatures (15, 20, 25, 30, and 40°C) and pH levels (4-9). After incubation, the cultures were centrifuged at 5000 rpm for 10 minutes, and the supernatant was analyzed for protease activity using the Folin reagent to estimate soluble tyrosine, with UV-VIS spectrophotometric readings taken at 660 nm (Sumantha *et al*., 2006). Protease activity was defined as the quantity of enzyme needed to release 1 µg of tyrosine per mL per minute under standard conditions.

Rearing of Insects

An artificial diet was prepared following the method described by Jones *et al*. (2002). *Galleria mellonella* larvae were collected from honeybee combs infested with the greater wax moth and reared in plastic jars (25 cm x 15 cm), each containing 5 mL of the artificial diet. The larvae were maintained under laboratory conditions at 30°C and 65% relative humidity until pupation. Once pupation was complete, the pupae were transferred to a separate container for adult emergence and mating. The resulting eggs were collected and placed on the artificial diet to maintain a uniform larval age for the experiment.

Determination of Mortality and Lethal Concentration (LC) Activity

Third-instar larvae, of similar size and weight (275-330 mg), were selected for the mortality experiments. Larvae were injected with 10 µL of one of the four spore concentrations into the left pro-leg using a 50 µL Hamilton syringe (Hamilton Company, UK). After treatment, the larvae were placed in Petri dishes lined with filter paper and kept at 30°C and 65% RH in the dark. Mortality was recorded after 24 hours based on criteria such as complete melanization, lack of response to touch, and inability to self-correct when rolled onto their back (Fuchs *et al*., 2010). LC50 and LC90 values were calculated using linear regression analysis.

Effect of Fungal Spores on Larval and Pupal Feeding

Nutritional indices were assessed by incorporating fungal spores into the artificial diet at different concentrations. Following the method described by Xie *et al*. (1996), 100 g of the artificial diet was mixed with 10,000 µL of one of the fungal spore suspensions. Distilled water mixed with the artificial diet was used as a control. Third-instar *G. mellonella* larvae were starved for 1 hour prior to the experiment, and then 10 larvae were placed in each container with 20 g of the prepared diet. Each experiment was replicated three times. Nutritional parameters, including the relative growth rate (RGR), relative consumption rate (RCR), efficiency of conversion of ingested food (ECI), and feeding deterrence index (FDI), were calculated according to Huang *et al*. (2000).

Histopathological deformation of the larval midgut

The infected larvae "treated with LC_{50}" were dissected to examine the histological effects of fungal infection. Larval tissues were fixed in 4% phosphate-buffered paraformaldehyde for 7 days, then dehydrated and embedded in Paraplast (Sigma-Aldrich). Sections 5 µm thick were prepared using a rotating microtome and mounted on polysine-coated glass slides. The sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol, and stained with hematoxylin for 7 minutes, followed by eosin for 15 minutes (Sigma-Aldrich). Calcofluor white staining was used to highlight chitinous structures, and tissues were analyzed using the confocal microscope (Perdoni *et al*., 2014).

Statistical Analysis

Data were represented as the mean ± standard error (SE). One-way analysis of variance (ANOVA) and Tukey's post hoc test were performed using SPSS software (version 26, SPSS Inc., Chicago) to compare mean differences. LC_{50} and LC_{90} values were calculated using Microsoft Excel (2021 version), and mortality percentages were corrected using Abbott's formula (Abbott, 1925).

RESULTS AND DISCUSSION

Effect of Different Factors on the Proteolytic Activity of *A. melleus*

Aspergillus melleus exhibited variable proteolytic activity depending on the incubation period, temperature, and pH. Maximum enzyme activity (2.62 U/mL) was observed after 72 hours of incubation (Figure 1), after which activity gradually declined, likely due to nutrient depletion or the accumulation of inhibitory by-products. Similarly, at 35°C, the enzyme displayed its highest protease activity (2.93 U/mL), demonstrating its thermal stability within this temperature range (Figure 2). Activity decreased outside this range, but was still significant between 25°C and 35°C, corroborating the findings of Mustefa Beyan *et al*. (2021), who reported similar optimal temperatures for *Aspergillus* species. The protease was also tested across a pH range of 4.0 to 9.0, with peak activity at pH 8 (Figure 3), confirming its alkaline nature. This is consistent with previous studies by Ito and Sugiura (1968), who reported pH 8 as optimal for *A. melleus* protease activity, and further supported by the stable performance of the enzyme across pH values between 7 and 10 (Mustefa Beyan *et al*., 2021).

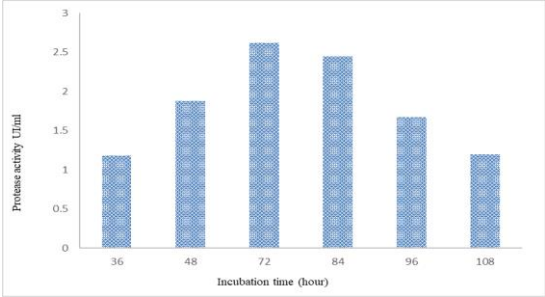

Figure 1. Effect of incubation period on protease activity of *A. melleus.*

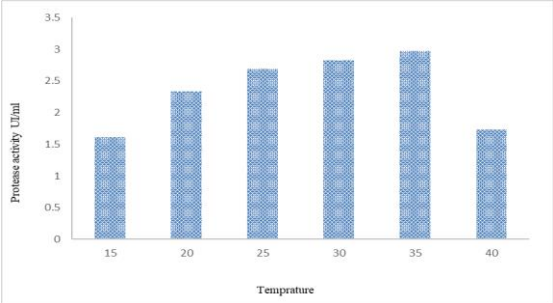

Figure 2. Effect of temperature on protease activity of *A. melleus.*

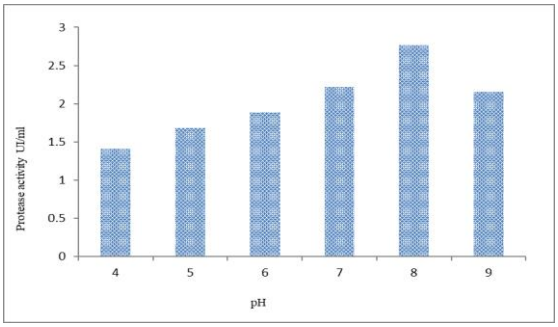

Figure 3. Effect of pH on protease activity of *A. melleus.*

Virulence of *A. melleus* Against Larvae and Pupae of *G. mellonella*

Data in Table 1 demonstrate the effect of *A. melleus* conidial concentrations on the duration of the larval and pupal developmental stages of *G. mellonella*. Statistical analysis revealed a significant extension in the developmental period of both larvae and pupae with increasing concentrations of fungal spores, compared to the control group. The control group exhibited the shortest developmental durations of 10.33±0.17 and 19.22±0.15 days for larvae and pupae, respectively. The highest conidial concentration of 1×10^9 conidia/mL resulted in the longest durations of 22.88±0.30 days for larvae and 29.57±0.29 days for pupae, with a gradual decrease in duration as spore concentrations reduced (Table 1). These results are consistent with findings by Hamama *et al.* (2021), who demonstrated increased larval mortality and extended pupal duration in *Culex pipiens* following exposure to fungal spores of *Beauveria bassiana* and *Metarhizium anisopliae*.

Table 1. Effect of *A. melleus* on Duration (in days) of Developmental stages of *G. mellonella.*

Conidial Concentration (conidia/mL)	Larval duration	Pupal duration
Control	10.33±0.17^{a}	19.22±0.15^{a}
1x10^{6}	11.67±0.17^{b}	21.33±0.33^{b}
1x10^{7}	14.00±0.17^{c}	23.63±0.50^{c}
1x10^{8}	17.20±0.29^{d}	25.90±0.28^{d}
1x10^{9}	22.88±0.30^{e}	29.57±0.29^{e}
F Value	459.632	145.272
P-value	<0.001	<0.001

Similarly, as Table 2 shows, *A. melleus* conidial concentrations caused significant larval and pupal mortality, with the highest concentration (1×10^9 conidia/mL) recording 93.10% total accumulated mortality.

Table 2. Corrected Mortality % of Conidial Concentrations of *A. melleus* Applied Against *G. mellonella* larvae.

Conidial Concentration (conidia/mL)	Larval mortality (%)	Pupal mortality (%)	Total mortality (%)	LC_{50} (conidia/mL)	LC_{90} (conidia/mL)
Control	0.0±0.0^{a}	0.0±0.00^{a}	0.00^{a}		
1x10^{6}	13.79±0.17^{ab}	0.00±00^{a}	13.79^{ab}		
1x10^{7}	34.48±0.10^{bc}	6.91±0.13^{ab}	41.39^{b}	1.9x10^{6}	2.5 x10^{8}
1x10^{8}	55.17±0.15^{bc}	10.35±0.15^{b}	65.52^{bc}		
1x10^{9}	75.86±21^{cd}	17.24±0.17^{b}	93.10^{c}		
F value	33.77	3.26	30.09		
P value	<0.001	<0.001	<0.001		

This finding aligns with previous research by Abdel-Rahman and Reda (2019), who found that higher concentrations of *M. anisopliae* resulted in increased mortality rates. Probit analysis was conducted to estimate the LC_{50} and LC_{90} values for *A. melleus* conidial concentrations against *G. mellonella* larvae.

The graph (Figure 4) shows the relationship between the log of the dose/concentration and the Probit response. The LC_{50} value was calculated as 1.3×10^6 conidia/mL, while the LC_{90} was 3.8×10^8 conidia/mL.

This indicates that higher spore concentrations significantly increase mortality in *G. mellonella* larvae, demonstrating the virulence of *A. melleus* at elevated concentrations.

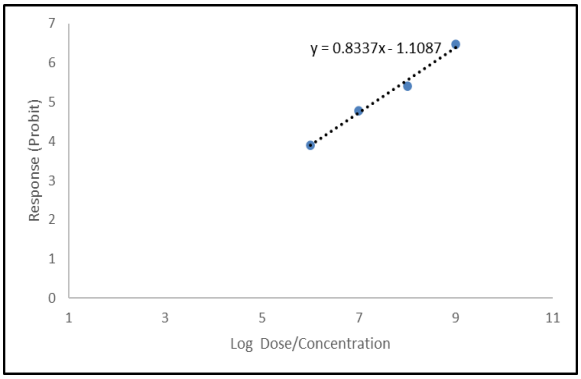

Figure 4. Probit analysis graph for determining LC_{50} and LC_{90} of *A. melleus* against *G. mellonella* larvae.

Impact of *A. melleus* on Food Consumption and Utilization

The nutritional indices, including the relative consumption rate (RCR), relative growth rate (RGR), and efficiency of conversion of ingested food (ECI), were significantly affected by *A. melleus* conidial concentrations (Table 3). The control group exhibited the highest RCR and RGR values of 0.25±0.003 g−1g−1d−1 and 1.51±0.024 g/g/day, respectively. However, with increasing concentrations of fungal spores, there was a marked decrease in both RCR and RGR. At 1×10^9 conidia/mL, the RCR dropped to 0.24±0.003, and the RGR to 0.43±0.016 g/g/day. The ECI also significantly decreased at the highest spore concentration (1×10^9 conidia/mL), while the feeding deterrence index (FDI) increased significantly, reaching 54.42% at the highest concentration (Table 3). These findings are consistent with research by Hussain *et al.* (2009), who found that spore suspensions of *B. bassiana* and *Isaria fumosorosea* significantly reduced RCR and RGR in insect larvae.

Table 3. Influence of *A. melleus* on Food Consumption and Utilization of *G. mellonella*

Conidial Concentration (conidia/mL)	Nutritional indices			
	RCR	RGR	ECI (%)	FDI (%)
control	0.25±.003[a]	1.51±.024[a]	16.667±.354[a]	39.980±1.45[a]
$1x10^6$	0.097±.004[b]	0.721±.011[b]	13.315± 0.280[b]	25.676±1.81[b]
$1x10^7$	0.096±.002[b]	0.711±0.011[b]	13.287±.303[b]	24.765±1.045[b]
$1x10^8$	0.064±.001[c]	0.568±.011[c]	11.373±.625[c]	33.949±1.37[c]
$1x10^9$	0.024±.003[d]	0.430±.016[d]	5.838±.750[d]	54.426±1.45[d]
F-Value	896.351	747.593	63.638	70.981
P-value	<001	<001	<001	<001

Melanization and Disease Progression

A degree of melanization was observed in infected larvae, as shown in Figure 5. Three days post-infection, the larvae exhibited initial signs of melanin pigmentation, which progressively spread throughout the entire body over the following seven days, culminating in death. This pattern is similar to observations by Durieux *et al.* (2021), who noted that fungal invasion by *A. fumigatus* led to complete melanization of the larva before death. The increase in melanization suggests an immune response, but the inability of the immune system to fully combat the infection resulted in significant mortality rates. According to Tojo *et al.* (2000), factors such as nutritional and thermal stress, or the pathogen's evasion of the immune system, can influence the extent of the melanization response in insect larvae.

Figure 5. Melanization of *G. mellonella* larvae as a visual indicator of fungal infection.
The series of images shows the progressive melanization of larvae infected with *A. melleus* over a seven-day period, starting from no visible melanization (far left) to complete melanization before death (far right).

Histological Effects of *A. melleus* Infection

Histological analysis of infected *G. mellonella* larvae revealed severe damage to the alimentary canal and other tissues (Figure 6). In the control larvae, the epithelial cells of the alimentary canal appeared normal, with intact membranes and nuclei. However, in larvae treated with higher concentrations of *A. melleus* conidia, there were clear signs of epithelial degradation. Vacuolation of epithelial cells, the loss of cell membrane integrity, and the presence of abnormal gastric caeca were prominent (Figure 6).

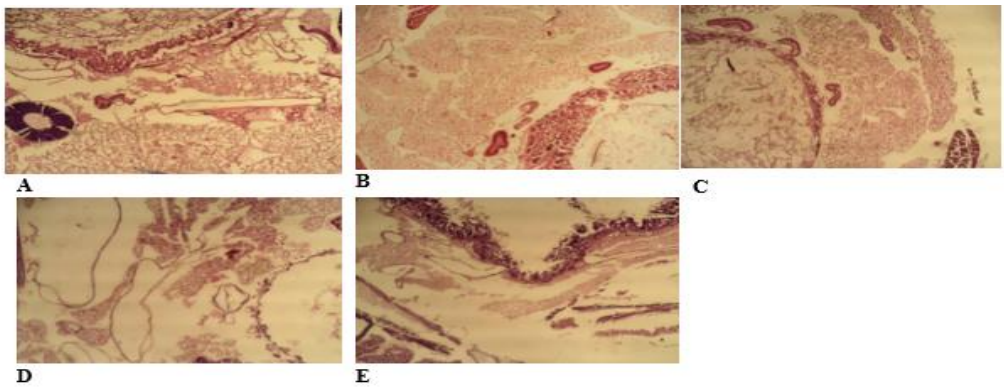

Figure 6. Histological sections of *G. mellonella* larvae infected with *A. melleus*.
A: Control showing normal epithelial cells and intact peritrophic membrane.
B: Early infection with vacuolated epithelial cells and detectable nuclei.
C: Moderate infection showing epithelial degradation and abnormal gastric caeca.
D: Severe degeneration of epithelial cells, thickened muscles, and abnormal silk gland.
E: Advanced infection with detached epithelial cells, thickened alimentary canal muscles, and degraded fat body.

As the infection progressed, the epithelial cells of the alimentary canal became highly vacuolated and detached from the basement membrane, while the alimentary canal muscles thickened. Gastric caeca displayed marked enlargement and abnormalities, and the fat body showed signs of degradation and irregular distribution. These findings align with Perdoni *et al*. (2014), who observed similar tissue degradation and immune responses in *G. mellonella* following fungal infection. The formation of nodules and the recruitment of hemocytes around the infection site were observed, reflecting the insect's immune response to the fungal invasion. Despite the immune response, the tissue damage caused by *A. melleus* resulted in the death of most larvae.

CONCLUSION

This study highlights the potent insecticidal activity of *A. melleus* alkaline protease against *G. mellonella* larvae. The protease exhibited significant biological effects, including increased larval and pupal mortality, extended developmental periods, and disruption of nutritional indices. Probit analysis revealed LC_{50} and LC_{95} values of 1.3×10^{6} and 3.8×10^{8} conidia/mL, respectively, demonstrating the virulence of *A. melleus* at higher concentrations. Histological analysis further confirmed severe tissue damage, particularly in the alimentary canal, including epithelial degradation, vacuolation, and abnormal muscle thickening. These findings indicate that *A. melleus* protease is a promising candidate for biological control strategies, particularly as a bio-insecticide for managing insect pests. Further studies on its field application and its potential effects on non-target organisms would provide greater insights into its practical use in integrated pest management programs.

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السمية والاضطرابات الغذائية الناتجة عن البروتييز القلوي لفطر *Aspergillus melleus* على يرقات الحشرات

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الملخص

تهدف هذه الدراسة إلى تقييم القدرة الإبادية لإنزيم البروتييز الذي ينتجه فطر *Aspergillus melleus* على يرقات فراشة الشمع الكبرى، وتركز على التأثيرات البيولوجية الهامة، والتي تتضمن زيادة معدلات الوفاة، التأخر في النمو، تدهور مؤشرات التغذية، والتغيرات المرضية بالأنسجة. وقد تم عزل الفطر من تربة منطقة الخطاطبة وإكثاره لإنتاج إنزيم البروتييز، حيث تم الوصول إلى الإنتاج الأمثل للإنزيم بعد 72 ساعة، عند درجة حموضة 8، ودرجة حرارة 35 مئوية. وقد أظهرت معدلات وفاة اليرقات والعذارى زيادة طردية بزيادة التركيز، حيث تم تحديد قيم LC_{50} و LC_{90} عند 1.9×10^6 و 2.5×10^8 بوغ/مل على التوالي. أدت تركيزات الفطر إلى تأخير ملحوظ في تطور اليرقات والعذارى وأحدثت تشوهات شكلية في الحشرة. وكشفت الدراسة التشريحية لليرقات المعاملة عن تلف في طبقة الخلايا الطلائية في المعي الأوسط لليرقات، مما أدى إلى ضعف امتصاص العناصر الغذائية. كما أظهرت مؤشرات التغذية انخفاضًا ملحوظًا في معدلات النمو والاستهلاك النسبي، بالإضافة إلى انخفاض في كفاءة تحويل الغذاء وزيادة في منع التغذية. تؤكد هذه النتائج على إمكانات إنزيم البروتييز الناتج من فطر *A. melleus* كمبيد حيوي للحشرات، حيث تمثل فراشة الشمع الكبرى نموذجًا جيدا لتقييم فعاليته ضد الحشرات.