In Press, Pre-Proof Version

Phenol-oxidase activity and haemocytes changes in *Helicoverpa armigera* Hübner infected by entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*

Akram Hatami[1*], Reza Farshbaf Pour Abad[1,2]*, Moosa Saber[1], and Rouhollah Motafakkerazad[3]

Abstract

Entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* are important and effective biocontrol agents against arthropod pests. Compared to chemical insecticides, insect pests do not easily develop resistance against these fungi. In this study, the lethal effects of exposure to *B. bassiana* and *M. anisopliae*, effects on phenol-oxidase activity, total haemocyte count, and changes in granulocytes and plasmatocytes were evaluated in 3rd instar larvae of *Helicoverpa armigera*. The LC_{50} values for *B. bassiana* and *M. anisopliae* were 0.795 $\times 10^6$, and 5.972 $\times 10^7$ spore ml^{-1}, respectively. LC_{30} and LC_{10} of either entomopathogenic fungi were injected into body of larvae, then, 24 and 48 hours after injection, their hemolymph was extracted. After 24 h the highest and lowest phenol-oxidase activity was observed in LC_{30} of *M. anisopliae*, and LC_{10} of *B. bassiana*, respectively. After 48 h of infection, phenol-oxidase activity increased in all treatments. At the LC_{30} of *M. anisopliae*, the highest phenol-oxidase activity was recorded, and other treatments also showed a significant difference compared to the control. Five types of hemocytes including prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes were identified in the hemolymph of larvae. The highest Total Hemocyte Count (THC) was recorded in LC_{30} *M. anisopliae* at 9 h after initial infection. The highest number of granulocytes and plasmatocytes were recorded 9 h after treatment in LC_{30} of *M. anisopliae* and LC_{30} of *B. bassiana* treatments. Our results showed that both fungi have the ability to affect phenol-oxidase enzyme activity and haemocytes. These microbial insecticides exhibited high potential for controlling the pest.

Keywords: Bioassay, Cotton bollworm, Microbial agents, Enzymes, Physiological effect.

Introduction

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is one of the most destructive pests of cotton and some other crops. To reduce the damage of this pest, insecticides from different

[1] Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, Islamic Republic of Iran.
[2] Department of Plant Protection, Faculty of Agriculture, Ege University, 35100 Bornova, Izmir, Turkiye.
[3] Department of Plant Sciences, Faculty of Natural Sciences, University of Tabriz, Tabriz, Islamic Republic of Iran.
Corresponding authors; e-mail: a.hatami0818@gmail.com and reza.farshbaf.pourabad@ege.edu.tr

chemical groups are used. Due to the increasing resistance of cotton bollworm against chemical insecticides, using the new and safe control measures is necessary. To control the pest, microbial pathogens including entomopathogenic fungi have been used in Integrated Pest Management Programs (IPM). Compared to chemical insecticides, microbial control agents are safe for humans and other non-target organisms, and they protect natural enemies and improve biodiversity (Mishra and Omkar, 2021). Several families of bacteria, fungi, viruses, and nematodes are used in biological control (Karim, 2000).

Insects, like other organisms, are usually infected by microbes including bacteria, viruses and fungi (Lacey *et al*., 2001). Entomopathogenic fungi are effective and environmentally safe control agents of the insect pests. High host specificity, negligible effects on non-target organisms, and ease of use are the advantages of these fungi (Singh *et al*., 2017). *Beauveria bassiana* and *Metarhizium anisopliae* are the most important entomopathogenic fungi used commercially (Mora *et al*., 2017).

Entomopathogenic fungi infect insects via host's cuticle. As insect pathogenic fungus enters the body of insect, the immune system of the insect both humoral and cellular confront against the pathogen. Insects destroy pathogenic bacteria and fungi by producing antimicrobial peptides (Kidanu and Hagos, 2020). Reactive intermediates of oxygen or nitrogen, as studied by Bogdan *et al*. (2000), and the Prophenol-Oxidase (PPO) activation system usually controls the coagulation and melanization of hemolymph (Cerenius and Söderhäll, 2021; Kanost and Gorman, 2008). Cellular immunity also causes phagocytosis, encapsulation and nodulation through hemocyte. Although insects have a strong immune system, pathogenic fungi can suppress it by releasing toxic substances into the hemolymph (Ferreira *et al*., 2023). In this way, entomopathogenic fungi easily cause septicemia. Finally, the fungus causes the death of the insect by multiplying in the homocell and consuming the nutrients of the host. Incubation period depends on various factors such as fungus species, virulence, host and its developmental stage. During this period, changes may be seen in the behavior and physiology of the insect, such as reduced feeding, weight, convulsions, imbalance, and paralysis (Deka *et al*., 2021). The cellular immune response of entomopathogenic fungi depends on circulating hemocyte. Plasmatocytes and granulocytes play the most important role against fungal spore. Plasmatocytes and granulocytes are responsible for destroying fungal spores through phagocytosis. After becoming infected, the cytoplasmic appendages of the hemocyte surrounding the spores increase. This enables the insect to effectively encapsulate and eliminate all of the spores using the phenol oxidase (Qu and Wang, 2018).

One of the key enzymes in the immune system of insects is phenol-oxyidase, which plays an important role in melanization. Phenol-oxidase exists in an inactive form, i.e. prophenol-oxidase. Prophenol-oxidase is converted to phenol-oxidase when pathogens enter the insect's body (Söderhäll and Cerenius, 1998). The activity of phenol-oxidase enzyme increases during the infection period (Mahmoud *et al*., 2015). One of the most important ways to improve the biological control effectiveness of entomopathogenic fungi is to determine the role of phenol-oxidase in insect immune response (Cao *et al*., 2016).

In this study, the efficacy of *B. bassiana* and *M. anisopliae* were evaluated on the 3rd instar larvae of the cotton bollworm, and then the effect of low-lethal concentrations (LC_{30} and LC_{10}) of each pathogen were investigated on phenol-oxidase enzyme activity and total haemocyte count.

Materials and Methods

Insect Rearing

Larvae were collected from cotton fields in Moghan, Iran. Larvae were reared on artificial diet. Artificial diet included: 206 g of cowpeas, 30 g of wheat germ, 35 g of yeast, 3.5 g of ascorbic acid, 1.1 g of sorbic acid, 2.2 g of methyl 4-hydroxybenzoate, 14 g of agar and 800 ml of distilled water (Shorey and Hale, 1965). The colony was reared in a growth room at 25±2°C, 50±5% RH and a photoperiod of 16L: 8D hours. The larvae were reared in rectangular containers with dimensions of 25×10×8 cm. Cylindrical containers (containing 10% honey water) were used to reared adult. The insects were reared for three generations and then 3rd instar larvae used for experiments.

Fungal Isolates

IR34-JS2 strain of *B. bassiana* and IR41-TT1 strain of *M. anisopliae* were obtained from the Department of Plant Protection, Faculty of Agriculture, Azarbaijan Shahid Madani University, Iran. These strains were collected and identified from the soils of several regions of Iran (Alizadeh, 2014) (Table 1). We cultured strains on PDA (Potato Dextrose Agar) and incubated them at temperature of 25±2°C.

Table 1. Details of entomopathogenic fungi strains used in bioassays.

Accession number	Source	Origin
IR34-JS2	Soil	Jiroft- Iran
IR41-TT1	Soil	Tabriz- Iran

Preparation of Suspension

15-day-old fungi colony was used to prepare suspension. 10 ml of 0.01% distilled water with Tween 80 was added to the Petri dishes. The spores were separated with a sterile brush. This suspension was vortexed for 3 minutes. The concentration of the suspension was determined using a hemocytometer (MARIENFELD BL2 Germany) (Faraji *et al*., 2013).

Bioassay

The lethal effects of *B. bassiana* and *M. anisopliae* were investigated on 3rd instar larvae of cotton bollworm. Larvae were immersed individually for 10 seconds into either of six different spore concentrations of fungal suspension (Safavi *et al*., 2010). Sterile distilled water was used as control. The suspension concentrations were 6×10^6, 3×10^6, 1.5×10^6, 0.75×10^6, 0.37×10^6, and 0.18×10^6 spore mL^{-1} for *B. bassiana*, and 32.5×10^7, 17.5×10^7, 8.5×10^7, 4.5×10^7, 2.5×10^7, and 1.5×10^7spore mL^{-1} for *M. anisopliae*. After immersion in the suspension, the larvae were transferred to individual sterile petri dishes (6cm, Biotest®) containing artificial diet. Experiments were performed in three replicates with 10 larvae in each replicate. Petri dishes were placed in a growth room with temperature of 25±2°C, photoperiod of 16:8 hours (light: darkness), and relative humidity of 50±5%. (The experiments were repeated twice under the same conditions). After eight days, the specimens were checked for mortality and results were recorded. During this 8-day period, symptoms of infection with pathogenic fungi were observed, including changes in behavior and physiology such as reduced feeding, convulsions, imbalance, and paralysis.

Injection of Microbial Agents

Low-Lethal Concentrations (LC_{30} and LC_{10}) of *B. bassiana* and *M. anisopliae* were used for injection into the larvae body. Ice cubes was used to immobilize the treated larvae. Then, one microliter of the suspensions (LC_{30} and LC_{10}) was injected (Hamilton syringe 10 µL. USA) into ventral body surface of larvae by Hamilton syringe (10 µL, USA). Distilled water was injected for control group (1 µL). After injection, the larvae were kept in laboratory conditions (Zhong *et al*., 2017).

Enzyme Assays

Leonard *et al*. (1985) method was used to determine the effect of *B. bassiana* and *M. anisopliae* on phenol-oxidase activity of third instar larvae. The hemolymph of larvae was collected 24 and 48 hours after infection. Due to the use of sublethal concentrations, it was necessary to allow sufficient time for the immune system to respond to even the lowest

concentration of the pathogen in the hemolymph. The third proleg of larvae was cut with a scalpel to collect hemolymph. To examine the activities of the PO, the hemolymph (500 µL) was centrifuged (Universal 320R) (10,000 rpm, 4°C, 5 minutes), and the supernatant was removed. Then, 100 µL of phosphate buffer (pH 7) was added. The extracted hemolymph was added to a 2 mL Eppendorf tube containing 0.02 g of Phenylthiourea (PTU) (Sigma-Aldrich®) (Anti-melanization). The sample was centrifuged at 12,000 rpm for 15 minutes, and the resulting liquid phase was utilized for the enzyme assay. To measure the activity of PO enzyme, 25 µL of hemolymph sample was added to 50 µL of 10 mM L-Dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich®) solution and 50 µL of phosphate buffer (pH 7) and incubated for 5 minutes at 30°C. Absorbance of the sample was recorded at 490 nm using ELISA reader, BioTek® ELX800 (Winooski, Vermont, USA).

Identification of hemocyte

For this purpose, 2 µL of hemolymph from larvae was placed on a sterile slide, and a smear was prepared. The smears were air dried, and then methanol (Merck®) and acetic acid (Merck®) (1:3 mL) were added. After drying, the hemolymph was treated with 10% Giemsa stain solution (Merck®) for 15 minutes, followed by a water rinse. The stained cells were identified using a light microscope at a magnification of 40x (Larvin and Strand, 2002; Giglio *et al*., 2008). Twenty larvae were used for each treatment.

Total Hemocyte Count

The LC_{30} and LC_{10} concentrations of either pathogen, was injected into the third instar larvae. After 3, 6, 9, and 12 hours, the prolegs was cut and the hemolymph was collected in a 2 mL microtube (BIO PLUS®). 20 µL of fresh hemolymph (1 larve) was diluted in 180 µL of anticoagulant buffer (98 mM NaOH, 146 mM NaCl, 17 mM ethylene diamine tetra acetic acid, 41 mM citric acid, pH 4.5) (Kalia *et al*., 2001). The THC (Total Hemocyte Count) was determined using a hemocytometer (Marienfeld® BL2) at 40X magnification (light microscope (Olympus®)). The number of hemocyte was counted in four corners and one central area [per cubic millimeter (mm^3)], and then calculated using the following formula of Jones (1962):

$$\text{THCs (Cells mm}^{-3}) = \frac{\text{Total number of cells counted} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Number of smallest squares counted}}$$

Dilution factor = 20; Depth factor= 10; Number of squares counted= 5.

This experiment was repeated four times with 10 larvae for each treatment (Istkhar and Chaubey, 2018).

Changes of Granulocytes and Plasmatocytes

The changes in plasmatocytes and granulocytes after treatment were determined using the Jones' formula.

Estimation of Protein Concentration

The protein concentration of the samples was determined according to the Bradford method (1976). The standard protein used in this study was BSA (Bovine Serum Albumin) (Sigma-Aldrich®). 10 µL of hemolymph sample was added to 190 µL of staining solutions. Then the samples were placed at room temperature (25°C) for 10 minutes. Optical densities were measured at 595 nm using ELISA reader.

Statistical Analysis

Data were analyzed using SPSS software (version 16). The means of the data, in the various experimental treatments were separated and statistically analyzed using one-way ANOVA, and Duncan's multiple range test at 5% probability level.

Results

Bioassays

The estimated LC_{50} values for *B. bassiana* and *M. anisopliae* were 0.795×10^6, and 5.972×10^7 spore ml^{-1}, respectively (Table 2). Our results showed that *B. bassiana* had higher toxicity on larvae of the cotton bollworm. Due to the non-significance of the x^2 factor, it is clear that the population is homogeneous and had the same response to the lethal effects of both fungi. In other words, the estimated values are not significantly different from the observed values (Table 2). According to the value of R^2 in the dose- response lines (Figure 1), there was high correlation between the concentration of fungi spores and the response of the population, so the tested population was homogeneous.

Table 2. Acute toxicity of two entomopathogenic fungi on 3rd instar larvae of *Helicoverpa armigera*.

Entomopathogenic fungus	x^2	df	*P*	Slope±SE	LC_{10} (spore mL^{-1})	LC_{30} (spore mL^{-1})	LC_{50} (spore mL^{-1})
Beauveria bassiana	0.38	4	0.98	1.23±0.2	0.072×10^6 (0.015×10^6-0.019×10^6)	0.297×10^6 (0.143×10^6-0.464×10^6)	0.795×10^6 (0.520×10^6-1.15×10^6)
Metarhizium anisopliae	0.57	4	0.97	1.22±0.22	0.531×10^7 (1.10×107-0.123×10^7)	2.218×10^7 (1.05×10^7-3.40×10^7)	5.972×10^7 (3.99×10^7-8.75×10^7)

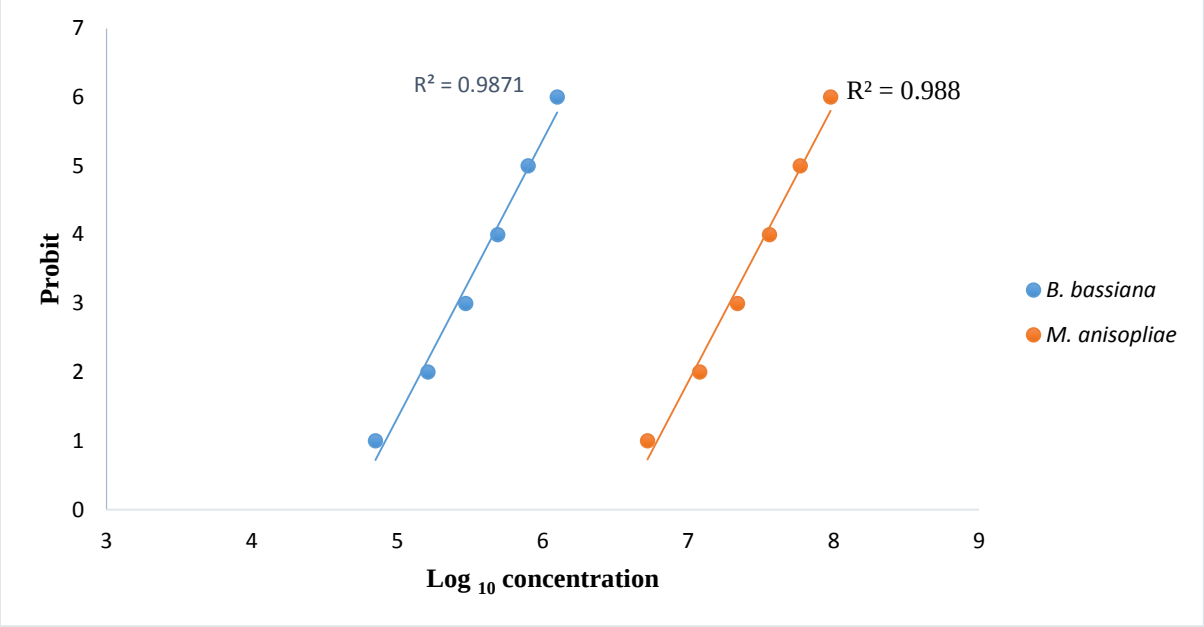

Figure 1. Concentration- response lines for *Beauveria bassiana* and *Metarhizium anisopliae* against the 3rd instar larvae of *Helicoverpa armigera*.

Phenol-Oxidase Activity

The results showed that the larvae injected with *B. bassiana* and *M. anisopliae* showed a significant increase in PO activity compared to the control group ($F_{4, 10} = 42.25$, $P < 0.01$). The results showed that the phenol-oxidase activity was significantly different from the control after 24 h in all treatments (Table 3).The highest PO activity was recorded in the LC_{30} treatment of *M. anisopliae*. However, no statistically significant difference was observed in phenol-oxidase activity between LC_{30} treatments of *M. anisopliae* and *B. bassiana.* The lowest phenol-oxidase activity was recorded 24 hours after infection of LC_{10} *B. bassiana* to larvae (Table 3). The results clearly indicated that a significant difference between the control and various concentrations after 48 hours of treatment ($F_{4, 10} = 45.69$, $P < 0.01$). The highest PO activity was observed in larvae treated with the LC_{30} of *M. anisopliae* in comparison with the other treatments (Table 3). There was no statistically significant difference between the LC_{10} treatments of both pathogens. According to the obtained results, the sub-lethal concentrations of the pathogen caused a significant increase in the activity of the PO enzyme, so PO activity has a direct relationship with pathogen concentration in hemolymph.

Table 3. Sublethal effects of entomopathogenic fungi on phenol-oxidase activity in third instar larvae of *Helicoverpa armigera.*

LC (spore mL^{-1})	Post injection (24 h) PO activity (U µl^{-1} min^{-1})±SE	Post injection (48 h) PO activity (U µl^{-1} min^{-1})±SE
LC_{10} *B. bassiana*	0.38 ± 0.04 b	0. 51 ± 0.03 b
LC_{10} *M. anisopliae*	0.53 ± 0.01 c	0. 62 ± 0.02 bc
LC_{30} *B. bassiana*	0.58 ± 0.01 cd	0.74 ± 0.05 c
LC_{30} *M. anisopliae*	0.62 ± 0.03 d	0.84 ± 0.03 d
Control	0.21 ± 0.01 a	0.22 ± 0.01 a

Similar small letters do not indicate significant differences in means (Duncan's test, P< 0.05).

Identification of Hemocyte

Five types of hemocyte have been identified in the hemolymph of cotton bollworms: prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherulocytes (Figure 2). Prohemocytes are the smallest cells, characterized by a large nucleus (Figure 2-a). Plasmatocytes are spindle-shaped and have two cytoplasmic appendages, which are crucial for adhesion to foreign agents (Ling *et al*., 2003) (Figure 2-c). Granulocytes are larger than plasmatocytes and have a cytoplasm filled with granules (Figure 2-d). These cells play a significant role in nodule formation (Tanaka and Yamakawa, 2011). Spherulocytes are relatively large cells with a large nucleus (Figure 2-b). Oenocytoids are circular cells with a lateral nucleus (Figure 2-e) and are one of the sources of phenol-oxidase enzyme production in Lepidoptera (Ling *et al*., 2005).

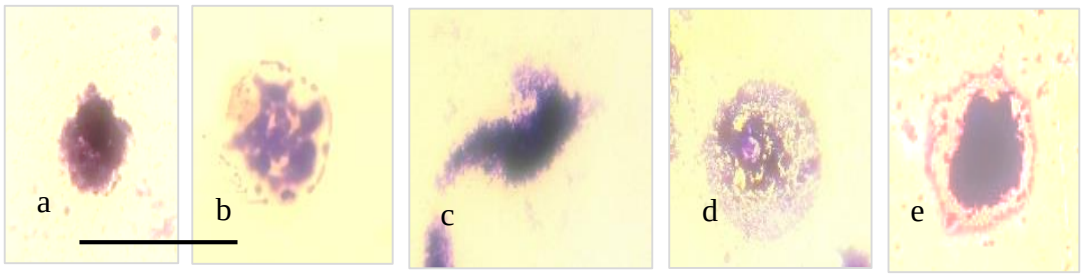

Figure 2. Hemocyte types of *Helicoverpa armigera* larvae by using Giemsa for light microscopic observations: a: Prohemocyte, b: Spherulocyte, c: plasmatocyte, d: Granulocyte, e: Oenocytoid (Scale bar= 10µm).

Total Hemocyte Count

Counting the number of Total Hemocyte Count (THC) showed that the injection of pathogens caused a change in the number of hemocyte ($F_{4, 15}$ = 1.03, P< 0.01). So that the number of hemocyte was increased in the first hours after the injection and then decreased. The results showed that at 3 hours post-injection, the number of hemocyte in the treatments was not significantly different from the control (Table 4). After 6 hours, there was a slight increase in the number of hemocyte in the treatment groups compared to the control. However, the more significant change was observed after 9 hours of treatment. The groups treated with LC_{30} *M. anisopliae* and LC_{30} *B. bassiana* had the highest number of hemocyte, with an average of 18×10^5 and 17.75×10^5 Cell mm^{-3}, respectively (Table 4). After 9 hours, there was a significant

difference in the LC_{10} treatment for both pathogens compared to the control. However, after 12 hours, there was a significant decrease in the number of hemocyte in the treatments compared to 9 hours. The LC_{10} treatments for both pathogens did not significant affected the total number of hemocyte compared to the control.

Table 4. The effect of entomopathogenic fungi on Total Hemocyte Count (THC) in the third instar larvae of *Helicoverpa armigera*.

Treatment (Spore mL^{-1})	3 h	6 h	9 h	12 h
	Cell×10^5 mm^{-3}	Cell×10^5 mm^{-3}	Cell×10^5 mm^{-3}	Cell×10^5 mm^{-3}
LC_{30} *B. bassiana*	10.25±0.62 a	13±0.40 b	17.75±1.1 c	15.50±0.64 b
LC_{30} *M. anisopliae*	10.50±0.64 a	12.25±0.85 ab	18±0.40 c	15.25±0.47 b
LC_{10} *B. bassiana*	9±0.40 a	11.50±0.64 ab	14.75±0.85 b	12.75±0.85 a
LC_{10} *M. anisopliae*	9.50±0.64 a	10.75±0.62 a	13.50±0.64 b	11.50±0.64 a
Control	9.50±0.64 a	10.75±0.47 a	10.75±0.85 a	11.50±0.64 a

Similar small letters do not indicate significant differences in means (Duncan's test, P< 0.05).

Changes in Granulocytes and Plasmatocytes

Three hours after injection, the highest number of granulocyte with an average of 6.75×10^3 cells mm^{-3} was observed in the *M. anisopliae* treatment compared to the other treatments ($F_{4, 10} = 5.1$, P< 0.01) (Table 5). After six hours, the number of granulocyte was increased in all treatments ($F_{4, 15} = 15.85$, P< 0.01). The treatment with LC_{30} *M. anisopliae* and LC_{30} *B. bassiana* caused the highest number of granulocyte (Table 5). After 9 hours, the highest number of granulocytes was recorded for the LC_{30} *M. anisopliae* treatment, with an average of 13.25×10^3cells /mm^3 ($F_{4, 15}$= 28.08, P< 0.01). However, after 12 hours, the number of granulocyte decreased in all treatments ($F_{4, 15}$= 19.84, P< 0.01).

Table 5. The effect of entomopathogenic fungi on granulocytes changes in the third instar larvae of *Helicoverpa armigera*.

Treatment (spore mL^{-1})	3h	6h	9h	12h
	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}
LC_{30} *B. bassiana*	6.25±0.25 bc	8.25±0.47 c	11.25±0.62 c	9.25±0.47 c
LC_{30} *M. anisopliae*	6.75±0.25 c	9±0.4 c	13.25±0.75 d	10.75±0.47 d
LC_{10} *B. bassiana*	5.75±0.25 ab	6.75±0.25 b	8.5±0.64 b	8±0.4 bc
LC_{10} *M. anisopliae*	5.75±0.25 ab	6.5±0.28 ab	9.25±0.25 b	7.25±0.47 b
Control	5±0.4 a	5.5±0.47 a	5.5±0.28 a	5.75±0.25 a

Similar small letters do not indicate significant differences in means (Duncan's test, P< 0.05).

The number of plasmatocytes was increased after the injection of pathogens (Table 6). 3 hours after the pathogen application the number of plasmatocyte in the LC_{30} treatments of both pathogens was significantly higher than the control ($F_{4, 15} = 5.35$, P< 0.01). Nine hours after infection, *B. bassiana* treatment showed the highest number of plasmatocyte at 11.75×10^3 Cell/mm^3, while the *M. anisopliae* treatment had the lowest number of plasmatocyte in the hemolymph ($F_{4, 15} = 14.06$, P< 0.01) (Table 6). However, after 12 hours, there was a decrease

in the number of plasmatocyte in all treatments, although they still were remained higher than the control group and showed a significant difference ($F_{4, 15}$= 13.03, P< 0.01).

Table 6. The effect of entomopathogenic fungi on plasmatocytes changes in the third instar larvae of *Helicoverpa armigera*.

Treatment (Spore mL^{-1})	3 h	6 h	9 h	12 h
	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}	Cell×10^3 mm^{-3}
LC_{30} *B. bassiana*	6.25±0.47 b	8.25±0.75 c	11.75±0.85 c	10±0.4 d
LC_{30} *M. anisopliae*	6.25±0.47 b	7.5±0.64 bc	10.25±0.62 bc	8.5±0.64 cd
LC_{10} *B. bassiana*	5±0.4 ab	5.75±0.25 bc	9.5±0.64 b	7.5±0.64 bc
LC_{10} *M. anisopliae*	5 ±0.4 ab	6.5±0.28 ab	9±0.57 b	6±0.4 ab
Control	3.75±0.47 a	4.5±0.64 a	4.75±0.75 a	5.25±0.47 a

Means in a column followed by similar small letters do not significantly different (Duncan's test, P< 0.05).

Estimation of Protein Concentration

The results showed that the injection of LC_{10} and LC_{30} concentrations of both fungi decreased the hemolymph protein concentration (Figure 3). 24 and 48 hours post-injection, larvae treated with LC_{30} *M. anisopliae* and LC_{10} *B. bassiana* had the lowest and highest protein concentrations, respectively, compared to the control.

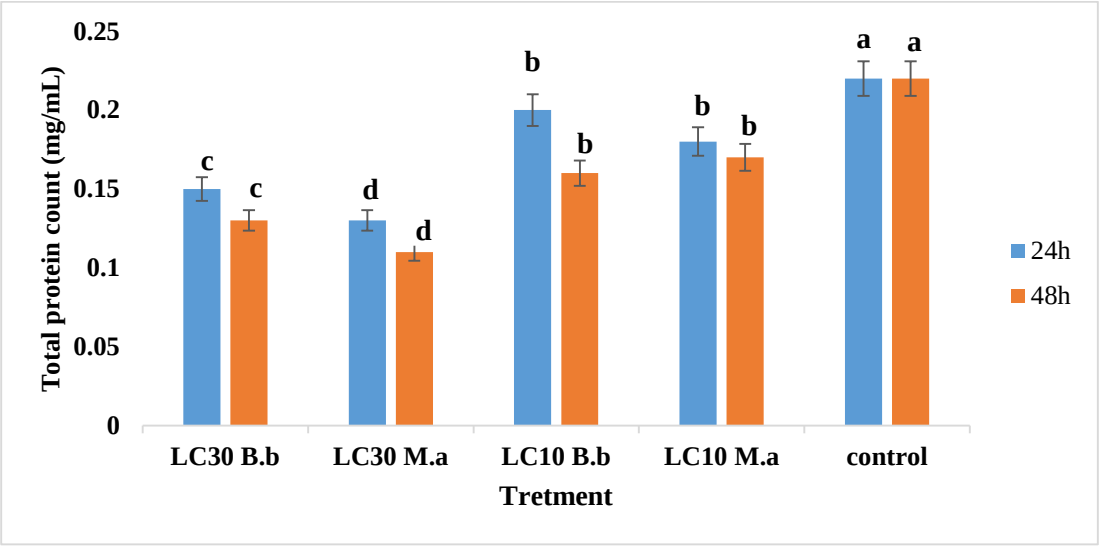

Figure 3. Total protein concentration of hemolymph sample 24 and 48h post-injection. Means followed by the same letter do not differ significantly (Duncan's test, P< 0.05). (B.b: *B. bassiana***,** M.a**:** *M. anisopliae*].

Discussion

Various studies have been conducted to investigate the virulence of microbial agents (Kalvandi *et al*., 2018; Dias *et al*., 2019). Our results showed that entomopathogenic fungi *B. bassiana* and *M. anisopliae* had effective control potential against cotton bollworm larvae. Souza *et al*. (2020) reported that isolates IBCB 1363 and IBCB 36 of *B. bassiana* and isolates IBCB 425 and ESALQ 860 of *M. anisopliae* caused the highest mortality against second-instar larvae of *H. armigera*. Petlamul *et al*. (2019) investigated 36 strains of *B. bassiana* on the third instar larvae of *H. armigera* and showed that the lowest tested concentration caused about 41%

mortality on larvae after five days. In our study, eight days after treatment, the lowest mortality was observed at the concentration of 0.18×10^6 spore ml^{-1}. Petlamul *et al*. (2019) evaluated the change in the activity of some enzymes and reported that the PO activity and glutathione-S-transferase was increased immediately after infection and decreased after 48 hours. In our study, PO activity was increased in the treatments compared to the control 24 and 48 h after infection. Insects increase the activity of PO enzyme after being attacked by pathogens to enhance their defense mechanisms against infections (Duffield *et al*., 2023). Bali and Kaur (2013) showed that PO activity in 3rd, 4th and 5th instar larvae of *Spodoptera litura* was affected when exposed *B. bassiana*. The activity of PO was increased in the 3rd and 4th instar larvae. No significant effect was observed on 5th instar larvae 24 hours after treatment.

Suppression of the host prophenol-oxidase activation pathway plays an essential role in the virulence of entomopathogenic fungi. According to the results of Liu *et al*. (2021), genetic manipulation in *B. bassiana* triggers the activation of an inhibitor, which in turn suppresses the activation of the host's prophenol oxidase. In our study, *B. bassiana* probably suppresses the activation of phenol oxidase. Therefore, the lowest activity was observed in the LC_{10} concentration of *B. bassiana* after 24 and 48 h of infection.

In the study conducted on the effect of two strains of *M. anisopliae* on *Locusta migratoria* L., it was found that the strain Ma IMI330189 was the most lethal against this pest. The results showed that the activitiy of PO was affected by *M. anisopliae*. The virulence of this strain is mainly due to its ability to penetrate into the insect body. In addition, yeast-like cells were observed in the infected locusts, which were transformed into hemolymph-derived hyphal bodies. They move in the hemolymph and cause damage (Cao *et al*., 2016). In the results obtained from our study, the PO enzyme in the treated larvae showed a significant increase compared to the control. The difference in pathogenicity and the change in the activity of enzymes is due to the difference in the penetration of the fungi and the dominance of hemocyte. When *B. bassiana* penetrates into the host body, the secondary metabolites of the fungus reduce the activity of phenol-oxidase. During evolution, entomopathogenic fungi have developed mechanisms to overcome the immune system of insects by changing cell size and their shape. This alteration enhances the absorption of substances from the insect. The second mechanism inactivates the host's immune response by releasing extracellular toxins (Tartar *et al*., 2005; Wahlman and Davidson, 1993; Suzuki *et al*., 1970). Zibaee *et al*. (2011) reported that treatment of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) with *B. bassiana* resulted in lower phenol-oxidase activity. Similar to the results of the present study, no significant difference in enzyme activity was observed in LC_{10} concentration compared to the control. The reason for

this decrease is the inhibition of the insect's immune system by secondary metabolites, which cause the death of the insect by overcoming the immune system. Therefore, attentions to the strains that produce effective secondary metabolites are important in select of effective strains for pest control (Shah and Pell, 2003).

Identification and classification of hemocyte is typically based on their morphological characteristics.

In previous studies, various types of hemocyte have been identified in the hemolymph of the *H. armigera*, including prohemocytes, plasmatocytes, granulocytes, spherulocytes, oenocytoids, and coagulocytes (Essawy *et al*, 1985; Gujar and Kalia, 2005). In a study by Kalia *et al* (2001), six types of hemocyte were identified in the hemolymph of cotton bollworms: prohemocytes, plasmatocytes, granulocytes, oenocytoids, spherulocytes, and adipohemocytes. Five types of hemocyte were identified in our research. In this study, the highest THC was recorded 9 hours after infection by entomopathogenic fungi. The results showed that the increase in hemocyte was directly related to the concentration of spores in the hemolymph. The highest THC was observed in the LC_{30} treatment of both pathogens. However, 12 hours after infection, the number of hemocyte showed a decreasing trend in the fungi treatments, although it was still significantly different from the control group.

Other studies have also confirmed the impact of pathogens on the increase of hemocyte. Changes in the hemocyte typically occurs within the first few hours after the pathogen enters the hemolymph. For instance, Khosravi *et al*. (2014) studied the impact of *B. bassiana* on the immune system of *Glyphodes pyloalis* Walker (Lepidoptera: Crambidae) larvae. They found that the number of hemocyte was increased following infection. But it was decreased after 12 and 24 hours. The number of granulocytes was increased in the early hours of infection and then decreased, which is consistent with the findings of our study. The decrease in hemocyte count in *Spodoptera exigua* larvae infected by *B. bassiana* was also documented by Hung and Boucias (1992). Zibaee and Malagoli (2014) evaluated the immune responses of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) to entomopathogenic fungi, *B. bassiana* and *M. anisopliae.* The results showed that larvae treated with *B. bassiana* and *M. anisopliae* had a significantly higher number of hemocyte at 3 and 6 hours after injection. The highest number of plasmatocytes was reported three hours after the injection of *B. bassiana*. After 6 hours, *M. anisopliae* caused a significant increase in plasmatocytes and granulocytes compared to the control group. In our study, the highest number of plasmatocytes and granulocytes were observed after 9 hours in both *B. bassiana* and *M. anisopliae* treatments.

Melanization plays a crucial role in cellular defense through the PO cascade. The onset of a fungal infection may have resulted in increased PO activity in treated insect. To protect the insect, melanin is produced to fight fungi. Changes in PO activity, which is an indicator of melanin production, have occurred as a result of haemocyte pathogenesis. The higher PO activity 48 post- infection suggested disintegration of hemocyte and release of PO into plasma that is correlated with decrease THC counts.

The results of our investigation showed that *B. bassiana* and *M. anisopliae* are effective against the 3rd instar larvae of cotton bollworm. Both fungi caused significant mortality in larvae. Considering the different mode of action of entomopathogenic fungi compared to chemical pesticides, they can be used for better control of this pest in the field. Studying changes in enzymes and enhancing the ability of pathogens to penetrate the insect body are crucial for effectively utilizing entomopathogenic fungi.

Acknowledgements

This paper has been published as part of a research project supported by the University of Tabriz Research Affairs Office, for which we are extremely grateful. We would like to express our gratitude to Dr. Hossein Sabouri, Associate Professor in the Faculty of Foreign Languages for carefully reviewing and improving the manuscript

References

1. Alizadeh, Z. 2014. Identification of entomopathogenic fungi from Khosrowshahr region based on phylogenetic analysis of ITS-rDNA sequence. M.Sc. thesis, Azarbaijan Shahid Madani University, 118 pp. (In Persian with English summary).
2. Bali, G.K., Kaur, S., Kour, B. 2013. Phenol-oxidase activity in haemolymph of *Spodoptera litura* (Fabricius) mediating immune responses challenge with entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillmin. *J. Entomol. Zool. Stud*., **1(6)**: 118-123.
3. Bogdan, C., Röllinghoff, M., Diefenbach, A. 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. *Curr. Opin. Immunol*., **12(1)**: 64-76.
4. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72:** 248–254.

5. Cao, G. Jia, M., Zhao, X., Wang, L., Tu, X. Wang, G., Nong, N., Zhang, Z. 2016. Different effects of *Metarhizium anisopliae* strains IMI330189 and IBC200614 on enzymes activities and hemocytes of *Locusta migratoria* L. *PLoS One*, **11(5)**: 1-8.
6. Duffield, K. R., Rosales, A. M., Muturi, E. J., Behle, R. W., Ramirez, J. L. 2023. Increased phenoloxidase activity constitutes the main defense strategy of *Trichoplusia ni* larvae against fungal entomopathogenic infections. *Insects*., 14(8), 667.
7. Cerenius, L. and Söderhäll, K. 2021. Immune properties of invertebrate phenol-oxidases. *Dev. Comp. Immunol*., **122**: 104098.
8. Deka, B., Baruah, C., Babu, A. 2021. Entomopathogenic microorganisms: their role in insect pest management. *Egypt. J. Biol. Pest Control.*, **31(1)**: 1-8.
9. De Souza, T.D., Fernandes, F.O., Sanches, A.C., Polanczyk, R.A., 2020. Sublethal effects of different fungal isolates on *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Egypt J Biol Pest Control.*, **30(1)**: 1-12.
10. Essawy, M., Maleville, A., Brehelin, M. 1985. The Hemocytes of *Heliothis armigera:* Ultrastructure, Functions, and Evolution in the Course of Larval Developmen. *J. Morphol.*, **186**: 255-264.
11. Faraji, S., Mehrvar, A., Shadmehri, A.D. 2013. Studies on the virulence of different isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metcsn.) Sorokin against Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). *Afr. J. Agric. Res*., **8**: 4157-4161.
12. Ferreira, J.M., and de Freitas Soares, F.E. 2023. Entomopathogenic fungi hydrolytic enzymes: A new approach to biocontrol?. *J. Nat. Pestic Res*., **3:** 1-12.
13. Giglio, A., Battistella, S., Talarico, F.F., Brandmayr, T.Z. and Giulianini, P.G. 2008. Circulating hemocytes from larvae and adults of *Carabus* (*Chaetocarabus*) *lefebvrei* Dejean 1826 (Coleoptera, Carabidae): cell types and their role in phagocytosis after in vivo artificial non-self-challenge. *Micron.*, **39**: 552-558.

Gujar, G.T. and Kalia, V.K. 2005. Hemocyte Diversity of the American Bollworm *Helicoverpa armigera. Phytoparasitica.*, **33(1)**: 17-27.

14. Hung, S. Y. and Boucias, D. G. 1992. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua*. *J. Invertebr. Pathol.*, 60(2):152-158.

15. Istkhar, R. and Chaubey, A.K. 2018. Challenging the larvae of *Helicoverpa armigera* and assessing the immune responses to nematode-bacterium complex. *Phytoparasitica*., **46**: 75-87.
16. Kalia, V., Chaudhari, S., Gujar, G. 2001. Changes in haemolymph constituents of American bollworm, *Helicoverpa armigera* Hübner infected with nucleopolyhedroviru. *Indian. J. Exp. Biol*., **39(11)**: 1123-1129.
Jones, J. C. 1962. Current concepts concerning insect hemocytes. *Am. Zool*, **2(2)**: .17 209-246.
18. Kalvnadi, E., Mirmoayedi, A., Alizadeh, M., Pourian, H.-R. 2018. Sub-lethal concentrations of the entomopathogenic fungus, *Beauveria bassiana* increase fitness costs of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol*., **158**: 32-42.
19. Kanost, M.R. and Gorman, M.J. 2008. Phenol-oxidases in insect immunity. *Insect immunology*, 1: 69-96.
20. Karim, S. 2000. Management of *Helicoverpa armigera*: a review and prospectus for Pakistan. *Pak. J. Biol. Sci*., **3(8)**: 1213-1222.
21. Kidanu, S. and Hagos, L. 2020. Research and application of entomopathogenic fungi as pest management option: a review. *J. Environ. Earth Sci*., **10(3)**: 31-39.
22. Khosravi, R., Sendi, J. J., Zibaee, A., Shokrgozar, M. A. 2014. Immune reactions of the lesser mulberry pyralid, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) to the entomopathogenic fungus, *Beauveria bassiana* (Bals.-Criv.) Vuill and two developmental hormones. *ISJ*., **11**: 11-21.
23. Lacey, L.A., Frutos, R., Kaya, H., Vail, P. 2001. Insect pathogens as biological control agents: do they have a future?. *Biol. Control*., **21(3)**: 230-248.
24. Lavine, M.D. and Strand, M.R. 2002. Insect hemocyte and their role in cellular immune responses. *Insect Biochem. Mol. Biol.*. **32**: 1237-1242.
25. Leonard, C., Söderhäll, K., Ratcliffe, N.A. 1985. Studies on prophenol-oxidase and protease activity of *Blaberus craniifer* haemocytes. *Insect Biochem*., **15(6)**: 803-810.
26. Ling, E., Shirai, K., Kanekatsu, R.., Kiguchi, K. 2005. Hemocyte differentiation in the hematopoietic organs of the silkworm, *Bombyx mori*: prohemocytes have the function of phagocytosis. *J. Cell Tissue Res*., **320**: 535-543.
27. Ling, E., Shirai, K., Kanekatsu, R., Kobayashi, Y., Tu, Z., Funayama, T. 2003. Why does hemocyte density rise at the wandering stage in the silkworm, *Bombyx mori*?. *J. Insect Biotechnol. Sericol*., **72**: 101-10.

28. Liu, Z.-C., Zhou, L., Wang, J.-L., Liu, X.-S. 2021. Expression of a phenol-oxidase cascade inhibitor enhances the virulence of the fungus *Beauveria bassiana* against the insect *Helicoverpa armigera*. *Dev. Comp. Immunol*., **117**: 1-7.
29. Mahmoud, D. Salem, D., Mo'men, S., Barakat, E., Salama, M. 2015. Purification and characterization of phenol-oxidase from immunized haemolymph of *Schistocerca gregaria*. *Afr. J. Biotechnol*., **14(44)**: 3027-3036.
30. Mingotti Dias, P., de Souza Loureiro, E., Amorim Pessoa, L.G., Mendes de Oliveira Neto, F., de Souza Tosta, RA., Teodoro, P.E. 2019. Interactions between fungal-infected *Helicoverpa armigera* and the predator *Chrysoperla externa*. Insects, **10(10)**: 1-11.
31. Mishra, G. and Omkar, 2021. Gram Pod Borer (*Helicoverpa armigera*). Polyphagous Pests of Crops: 311-348.
32. Mora, M.A.E., Castilho, A.M.C., Fraga, M.E. 2018. Classification and infection mechanism of entomopathogenic fungi. *Arq. Inst. Biol., Sao Paulo*., **8:** 1-10.
33. Petlamul, W., Boukaew, S., Hauxwell, C., Prasertsan, P. 2019. Effects on detoxification enzymes of *Helicoverpa armigera* (Lepidoptera: Noctuidae) infected by *Beauveria bassiana* spores and detection of its infection by PCR. *Sci. Asia*., **45**: 581–588.
34. Qu, S. and Wang, S. 2018. Interaction of entomopathogenic fungi with the host immune system. *Dev Comp Immunol*., 1-8.
35. Safavi, S., Kharrazi, A., Rasoulian, G.R., Bandani, A. 2010. Virulence of some isolates of entomopathogenic fungus, *Beauveria bassiana* on *Ostrinia nubilalis* (Lepidoptera: Pyralidae) *larvae. J. Agric. Sci. Technol*., **12(1)**: 13-21.
36. Shah, P. A. and J. K. Pell. 2003. Entomopathogenic fungi as biological control agents. *Appl. Microbiol. Biotechnol*., 61(5): 413-423.
37. Shorey, H. and Hale, R. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol*., **58(3)**: 522-524.
38. Singh, D., Raina, T.K., Singh, J. 2017. Entomopathogenic fungi: An effective biocontrol agent for management of insect populations naturally. *J. Appl. Pharm. Sci. Res*., **9(6)**: 833.
39. Söderhäll, K. and Cerenius, L. 1998. Role of the prophenol-oxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol*., **10(1)**: 23-28.
40. Suzuki, A., Taguchi, H., Tamura, S. 1970. Isolation and structure elucidation of three new insecticidal cyclodepsipeptides, destruxins C and D and desmethyldestruxin B, produced by *Metarrhizium anisopliae*. *Agric. Biol. Chem*., **34(5)**: 813-816.

41. Tanaka, H., and Yamakawa, M. 2011. Regulation of the innate immune responses in the silkworm, *Bombyx mori. Invertebrate surviv. j*., **8**: 59-69.
42. Tartar, A., Shapiro, A.M., Scharf, D.W., Boucias, D.G. 2005. Differential expression of chitin synthase (CHS) and glucan synthase (FKS) genes correlates with the formation of a modified, thinner cell wall in in vivo-produced *Beauveria bassiana* cells. *Mycopathologia*., **160**: 303-314.
43. Wahlman, M. and Davidson, B.S. 1993. New destruxins from the entomopathogenic fungus *Metarhizium anisopliae*. *J. Nat. Prod*., **56(4)**: 643-647.
44. Zhong, K., Liu, Z.C., Wang, J.L., Liu, X.S. 2017. The entomopathogenic fungus *Nomuraea rileyi* impairs cellular immunity of its host *Helicoverpa armigera*. *Arch. Insect Biochem. Physiol*., **96(1)**: 1-10.
45. Zibaee, A., Bandani, A.R., Talaei-Hassanlouei, R., Malagoli, D. 2011. Cellular immune reactions of the sunn pest, *Eurygaster integriceps*, to the entomopathogenic fungus, *Beauveria bassiana* and its secondary metabolites. *J. Insect Sci*., **11(1)**: 1-16.
46. Zibaee, A. and Malagoli, D. (2014). Immune response of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to different entomopathogenic fungi. *Bull. Entomol. Res.*, **104(2)**: 155-163.

فعالیت فنل اکسیداز و تغییرات هموسیت ها در *Helicoverpa armigera* Hübner آلوده به قارچ های بیماری گر، *Beauveria bassiana* و *Metarhizium anisopliae*

اکرم حاتمی، رضا فرشباف پورآباد، موسی صابر، و روح الله متفکرآزاد

چکیده

قارچ های بیمارگر حشرات، *Beauveria bassiana* و *Metarhizium anisopliae* از عوامل بیولوژیکی مهم و موثر در برابر آفات بندپایان هستند. در مقایسه با حشره کش های شیمیایی، آفات حشرات به راحتی در برابر این قارچ ها مقاومت نمی کنند. در این مطالعه، اثرات کشنده قرار گرفتن در معرض *B. bassiana* و *M. anisopliae*، اثرات بر فعالیت فنل اکسیداز، تعداد کل هموسیت ها و تغییرات در گرانولوسیت ها و پلاسماتوسیت ها در لاروهای سن 3 *Helicoverpa armigera* مورد بررسی قرار گرفت. مقادیر LC50 برای *B. bassiana* و *M. anisopliae* به ترتیب 0.795×106 و 5.972×107 اسپور ml^{-1} بود. LC30 و LC10 هر یک از قارچ های بیمارگر حشرات به بدن لارو تزریق شد، سپس 24 و 48 ساعت پس از تزریق، همولنف آنها استخراج شد. پس از 24 ساعت بیشترین و کمترین فعالیت فنل اکسیداز به ترتیب در LC30 *M. anisopliae* و LC10 *B. bassiana* مشاهده شد. پس از 48 ساعت آلودگی، فعالیت فنل اکسیداز در تمام تیمارها افزایش یافت. در LC30 *M. anisopliae*، بیشترین فعالیت فنل اکسیداز ثبت شد و سایر تیمارها نیز تفاوت معنی داری را نسبت به شاهد نشان دادند. پنج نوع هموسیت شامل پروهموسیت ها، پلاسماتوسیت ها، گرانولوسیت ها، انوسیتوئیدها و اسفرولوسیت ها در همولنف لارو شناسایی شد. بالاترین تعداد کل هموسیت (THC) در LC30 *M. anisopliae* در 9 ساعت پس از عفونت اولیه ثبت شد. بیشترین تعداد گرانولوسیت و پلاسماتوسیت 9 ساعت پس از تیمار، در تیمارهای LC30 *M. anisopliae* و LC30 *B. bassiana* ثبت شد. نتایج ما نشان داد که هر دو قارچ توانایی تأثیرگذاری بر فعالیت آنزیم فنل اکسیداز و هموسیت ها را دارند. این حشره کش های میکروبی پتانسیل بالایی برای کنترل آفت از خود نشان دادند.