

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

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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 LC₅₀ values for *B. bassiana* and *M. anisopliae* were 0.795×10^6 , and 5.972×10^7 spore ml⁻¹, respectively. LC₃₀ and LC₁₀ 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 LC₃₀ of *M. anisopliae*, and LC₁₀ of *B. bassiana*, respectively. After 48 h of infection, phenol-oxidase activity increased in all treatments. At the LC₃₀ 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 LC₃₀ *M. anisopliae* at 9 h after initial infection. The highest number of granulocytes and plasmatocytes were recorded 9 h after treatment in LC₃₀ of *M. anisopliae* and LC₃₀ 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

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

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

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

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

72 In this study, the efficacy of *B. bassiana* and *M. anisopliae* were evaluated on the 3rd instar
73 larvae of the cotton bollworm, and then the effect of low-lethal concentrations (LC₃₀ and LC₁₀)
74 of each pathogen were investigated on phenol-oxidase enzyme activity and total haemocyte
75 count.

76

77 **Materials and Methods**

78 **Insect Rearing**

79 Larvae were collected from cotton fields in Moghan, Iran. Larvae were reared on artificial
80 diet. Artificial diet included: 206 g of cowpeas, 30 g of wheat germ, 35 g of yeast, 3.5 g of
81 ascorbic acid, 1.1 g of sorbic acid, 2.2 g of methyl 4-hydroxybenzoate, 14 g of agar and 800
82 ml of distilled water (Shorey and Hale, 1965). The colony was reared in a growth room at
83 25±2°C, 50±5% RH and a photoperiod of 16L: 8D hours. The larvae were reared in rectangular
84 containers with dimensions of 25×10×8 cm. Cylindrical containers (containing 10% honey
85 water) were used to reared adult. The insects were reared for three generations and then 3rd
86 instar larvae used for experiments.

87

88 **Fungal Isolates**

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

94

95

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

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

96

97

98

99 **Preparation of Suspension**

100 15-day-old fungi colony was used to prepare suspension. 10 ml of 0.01% distilled water with
101 Tween 80 was added to the Petri dishes. The spores were separated with a sterile brush. This
102 suspension was vortexed for 3 minutes. The concentration of the suspension was determined
103 using a hemocytometer (MARIENFELD BL2 Germany) (Faraji *et al.*, 2013).

104 **Bioassay**

105 The lethal effects of *B. bassiana* and *M. anisopliae* were investigated on 3rd instar larvae of
106 cotton bollworm. Larvae were immersed individually for 10 seconds into either of six different
107 spore concentrations of fungal suspension (Safavi *et al.*, 2010). Sterile distilled water was used
108 as control. The suspension concentrations were 6×10^6 , 3×10^6 , 1.5×10^6 , 0.75×10^6 , 0.37×10^6 ,
109 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 ,
110 and 1.5×10^7 spore mL⁻¹ for *M. anisopliae*. After immersion in the suspension, the larvae were
111 transferred to individual sterile petri dishes (6cm, Biotest[®]) containing artificial diet.
112 Experiments were performed in three replicates with 10 larvae in each replicate. Petri
113 dishes were placed in a growth room with temperature of $25 \pm 2^\circ\text{C}$, photoperiod of 16:8 hours
114 (light: darkness), and relative humidity of $50 \pm 5\%$. (The experiments were repeated twice under
115 the same conditions). After eight days, the specimens were checked for mortality and results
116 were recorded. During this 8-day period, symptoms of infection with pathogenic fungi were
117 observed, including changes in behavior and physiology such as reduced feeding, convulsions,
118 imbalance, and paralysis.

120 **Injection of Microbial Agents**

121 Low-Lethal Concentrations (LC₃₀ and LC₁₀) of *B. bassiana* and *M. anisopliae* were used for
122 injection into the larvae body. Ice cubes was used to immobilize the treated larvae. Then, one
123 microliter of the suspensions (LC₃₀ and LC₁₀) was injected (Hamilton syringe 10 μL, USA)
124 into **ventral** body surface of larvae by Hamilton syringe (10 μL, USA). Distilled water was
125 injected for control group (1 μL). After injection, the larvae were kept in laboratory conditions
126 (Zhong *et al.*, 2017).
127

128 **Enzyme Assays**

129 Leonard *et al.* (1985) method was used to determine the effect of *B. bassiana* and *M.*
130 *anisopliae* on phenol-oxidase activity of third instar larvae. The hemolymph of larvae was
131 collected 24 and 48 hours after infection. Due to the use of sublethal concentrations, it was
132 necessary to allow sufficient time for the immune system to respond to even the lowest
133

134 concentration of the pathogen in the hemolymph. The third proleg of larvae was cut with a
135 scalpel to collect hemolymph. To examine the activities of the PO, the hemolymph (500 μ L)
136 was centrifuged (Universal 320R) (10,000 rpm, 4°C, 5 minutes), and the supernatant was
137 removed. Then, 100 μ L of phosphate buffer (pH 7) was added. The extracted hemolymph was
138 added to a 2 mL Eppendorf tube containing 0.02 g of Phenylthiourea (PTU) (Sigma-Aldrich®)
139 (Anti-melanization). The sample was centrifuged at 12,000 rpm for 15 minutes, and the
140 resulting liquid phase was utilized for the enzyme assay. To measure the activity of PO enzyme,
141 25 μ L of hemolymph sample was added to 50 μ L of 10 mM L-Dihydroxyphenylalanine (L-
142 DOPA) (Sigma-Aldrich®) solution and 50 μ L of phosphate buffer (pH 7) and incubated for 5
143 minutes at 30°C. Absorbance of the sample was recorded at 490 nm using ELISA reader,
144 BioTek® ELX800 (Winooski, Vermont, USA).

145

146 **Identification of hemocyte**

147 For this purpose, 2 μ L of hemolymph from larvae was placed on a sterile slide, and a smear
148 was prepared. The smears were air dried, and then methanol (Merck®) and acetic acid (Merck®)
149 (1:3 mL) were added. After drying, the hemolymph was treated with 10% Giemsa stain solution
150 (Merck®) for 15 minutes, followed by a water rinse. The stained cells were identified using a
151 light microscope at a magnification of 40x (Larvin and Strand, 2002; Giglio *et al.*, 2008).
152 Twenty larvae were used for each treatment.

153

154 **Total Hemocyte Count**

155 The LC₃₀ and LC₁₀ concentrations of either pathogen, was injected into the third instar larvae.
156 After 3, 6, 9, and 12 hours, the prolegs was cut and the hemolymph was collected in a 2 mL
157 microtube (BIO PLUS®). 20 μ L of fresh hemolymph (1 larve) was diluted in 180 μ L of
158 anticoagulant buffer (98 mM NaOH, 146 mM NaCl, 17 mM ethylene diamine tetra acetic acid,
159 41 mM citric acid, pH 4.5) (Kalia *et al.*, 2001). The THC (Total Hemocyte Count) was
160 determined using a hemocytometer (Marienfeld® BL2) at 40X magnification (light microscope
161 (Olympus®)). The number of hemocyte was counted in four corners and one central area [per
162 cubic millimeter (mm^3)], and then calculated using the following formula of Jones (1962):

$$163 \quad \text{THCs (Cells mm}^{-3}\text{)} = \frac{\text{Total number of cells counted} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Number of smallest squares counted}}$$

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

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

167

168 **Changes of Granulocytes and Plasmatocytes**

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

171

172 **Estimation of Protein Concentration**

173 The protein concentration of the samples was determined according to the Bradford method
174 (1976). The standard protein used in this study was BSA (Bovine Serum Albumin) (Sigma-
175 Aldrich®). 10 µL of hemolymph sample was added to 190 µL of staining solutions. Then the
176 samples were placed at room temperature (25°C) for 10 minutes. Optical densities were
177 measured at 595 nm using ELISA reader.

178

179 **Statistical Analysis**

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

183

184 **Results**

185 **Bioassays**

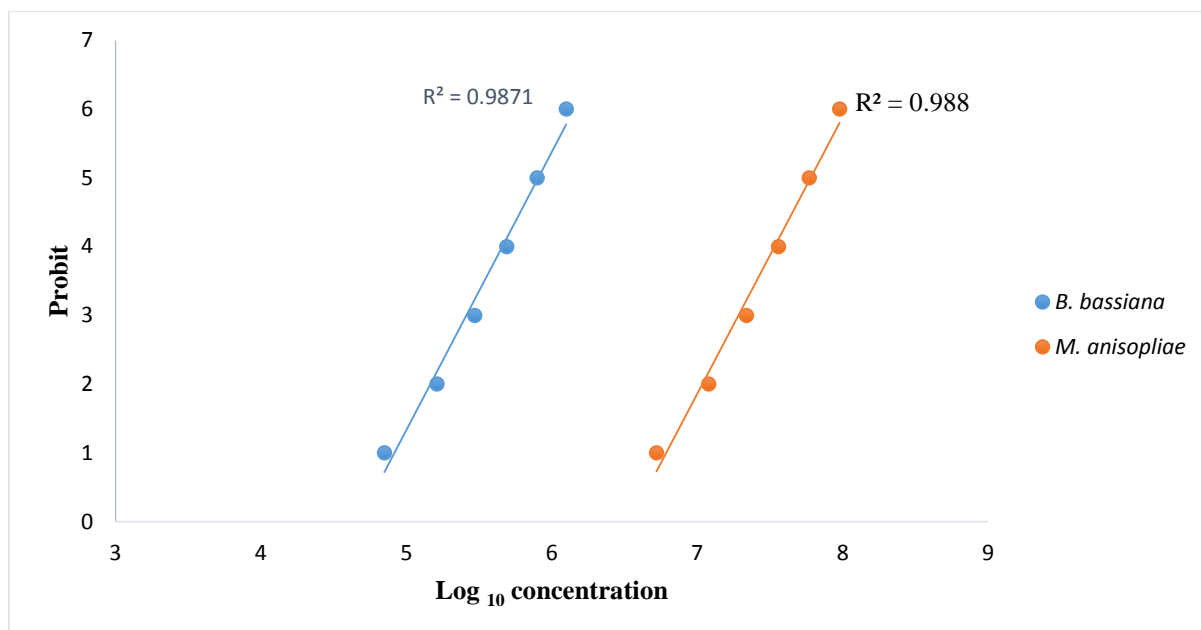
186 The estimated LC₅₀ values for *B. bassiana* and *M. anisopliae* were 0.795×10⁶, and 5.972×10⁷
187 spore ml⁻¹, respectively (Table 2). Our results showed that *B. bassiana* had higher toxicity on
188 larvae of the cotton bollworm. Due to the non-significance of the x^2 factor, it is clear that the
189 population is homogeneous and had the same response to the lethal effects of both fungi. In
190 other words, the estimated values are not significantly different from the observed values
191 (Table 2). According to the value of R² in the dose- response lines (Figure 1), there was high
192 correlation between the concentration of fungi spores and the response of the population, so
193 the tested population was homogeneous.

194

195 **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)
<i>Beauveria bassiana</i>	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)
<i>Metarhizium anisopliae</i>	0.57	4	0.97	1.22±0.22	0.531×10 ⁷ (1.10×10 ⁷ - 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)

196



197
198 **Figure 1.** Concentration- response lines for *Beauveria bassiana* and *Metarhizium anisopliae* against the 3rd
199 instar larvae of *Helicoverpa armigera*.

200 201 **Phenol-Oxidase Activity**

202 The results showed that the larvae injected with *B. bassiana* and *M. anisopliae* showed a
203 significant increase in PO activity compared to the control group ($F_{4, 10} = 42.25$, $P < 0.01$). The
204 results showed that the phenol-oxidase activity was significantly different from the control after
205 24 h in all treatments (Table 3). The highest PO activity was recorded in the LC₃₀ treatment of
206 *M. anisopliae*. However, no statistically significant difference was observed in phenol-oxidase
207 activity between LC₃₀ treatments of *M. anisopliae* and *B. bassiana*. The lowest phenol-oxidase
208 activity was recorded 24 hours after infection of LC₁₀ *B. bassiana* to larvae (Table 3). The
209 results clearly indicated that a significant difference between the control and various
210 concentrations after 48 hours of treatment ($F_{4, 10} = 45.69$, $P < 0.01$). The highest PO activity was
211 observed in larvae treated with the LC₃₀ of *M. anisopliae* in comparison with the other
212 treatments (Table 3). There was no statistically significant difference between the LC₁₀
213 treatments of both pathogens. According to the obtained results, the sub-lethal concentrations
214 of the pathogen caused a significant increase in the activity of the PO enzyme, so PO activity
215 has a direct relationship with pathogen concentration in hemolymph.

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224 **Table 3.** Sublethal effects of entomopathogenic fungi on phenol-oxidase activity in third instar larvae of
 225 *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 <i>B. bassiana</i>	0.38 ± 0.04 b	0.51 ± 0.03 b
LC ₁₀ <i>M. anisopliae</i>	0.53 ± 0.01 c	0.62 ± 0.02 bc
LC ₃₀ <i>B. bassiana</i>	0.58 ± 0.01 cd	0.74 ± 0.05 c
LC ₃₀ <i>M. anisopliae</i>	0.62 ± 0.03 d	0.84 ± 0.03 d
Control	0.21 ± 0.01 a	0.22 ± 0.01 a

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

227

228 Identification of Hemocyte

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

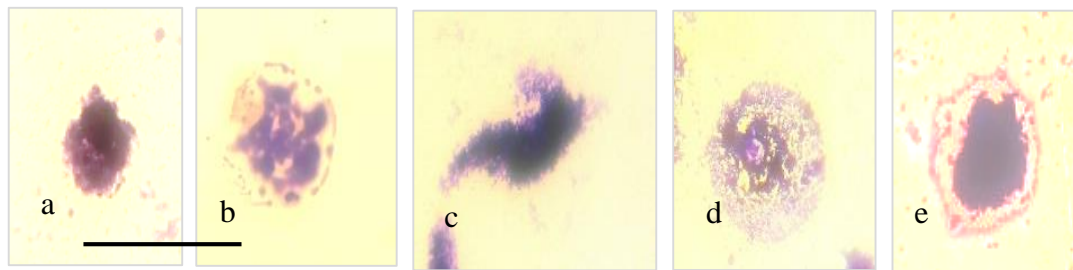
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244 **Figure 2.** Hemocyte types of *Helicoverpa armigera* larvae by using Giemsa for light microscopic observations:
 245 a: Prohemocyte, b: Spherulocyte, c: plasmatocyte, d: Granulocyte, e: Oenocytoid (Scale bar= 10μm).

246

247 Total Hemocyte Count

248 Counting the number of Total Hemocyte Count (THC) showed that the injection of pathogens
 249 caused a change in the number of hemocyte ($F_{4, 15} = 1.03, P < 0.01$). So that the number of
 250 hemocyte was increased in the first hours after the injection and then decreased. The results
 251 showed that at 3 hours post-injection, the number of hemocyte in the treatments was not
 252 significantly different from the control (Table 4). After 6 hours, there was a slight increase in
 253 the number of hemocyte in the treatment groups compared to the control. However, the more
 254 significant change was observed after 9 hours of treatment. The groups treated with LC₃₀ *M.*
 255 *anisopliae* and LC₃₀ *B. bassiana* had the highest number of hemocyte, with an average of
 256 18×10^5 and 17.75×10^5 Cell mm⁻³, respectively (Table 4). After 9 hours, there was a significant

257 difference in the LC₁₀ treatment for both pathogens compared to the control. However, after 12
 258 hours, there was a significant decrease in the number of hemocyte in the treatments compared
 259 to 9 hours. The LC₁₀ treatments for both pathogens did not significant affected the total number
 260 of hemocyte compared to the control.

261
 262 **Table 4.** The effect of entomopathogenic fungi on Total Hemocyte Count (THC) in the third instar larvae of
 263 *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 <i>B. bassiana</i>	10.25±0.62 a	13±0.40 b	17.75±1.1 c	15.50±0.64 b
LC ₃₀ <i>M. anisopliae</i>	10.50±0.64 a	12.25±0.85 ab	18±0.40 c	15.25±0.47 b
LC ₁₀ <i>B. bassiana</i>	9±0.40 a	11.50±0.64 ab	14.75±0.85 b	12.75±0.85 a
LC ₁₀ <i>M. anisopliae</i>	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

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

265

266 **Changes in Granulocytes and Plasmatocytes**

267 Three hours after injection, the highest number of granulocyte with an average of 6.75×10³
 268 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
 269 treatments ($F_{4, 15} = 15.85, P < 0.01$). The treatment with LC₃₀ *M. anisopliae* and LC₃₀ *B.*
 270 *bassiana* caused the highest number of granulocyte (Table 5). After 9 hours, the highest number
 271 of granulocytes was recorded for the LC₃₀ *M. anisopliae* treatment, with an average of
 272 13.25×10³cells /mm³ ($F_{4, 15} = 28.08, P < 0.01$). However, after 12 hours, the number of
 273 granulocyte decreased in all treatments ($F_{4, 15} = 19.84, P < 0.01$).
 274

275
 276 **Table 5.** The effect of entomopathogenic fungi on granulocytes changes in the third instar larvae of *Helicoverpa*
 277 *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 <i>B. bassiana</i>	6.25±0.25 bc	8.25±0.47 c	11.25±0.62 c	9.25±0.47 c
LC ₃₀ <i>M. anisopliae</i>	6.75±0.25 c	9±0.4 c	13.25±0.75 d	10.75±0.47 d
LC ₁₀ <i>B. bassiana</i>	5.75±0.25 ab	6.75±0.25 b	8.5±0.64 b	8±0.4 bc
LC ₁₀ <i>M. anisopliae</i>	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

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

279

280 The number of plasmatocytes was increased after the injection of pathogens (Table 6). 3 hours
 281 after the pathogen application the number of plasmatocyte in the LC₃₀ treatments of both
 282 pathogens was significantly higher than the control ($F_{4, 15} = 5.35, P < 0.01$). Nine hours after
 283 infection, *B. bassiana* treatment showed the highest number of plasmatocyte at 11.75×10³
 284 Cell/mm³, while the *M. anisopliae* treatment had the lowest number of plasmatocyte in the
 285 hemolymph ($F_{4, 15} = 14.06, P < 0.01$) (Table 6). However, after 12 hours, there was a decrease

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

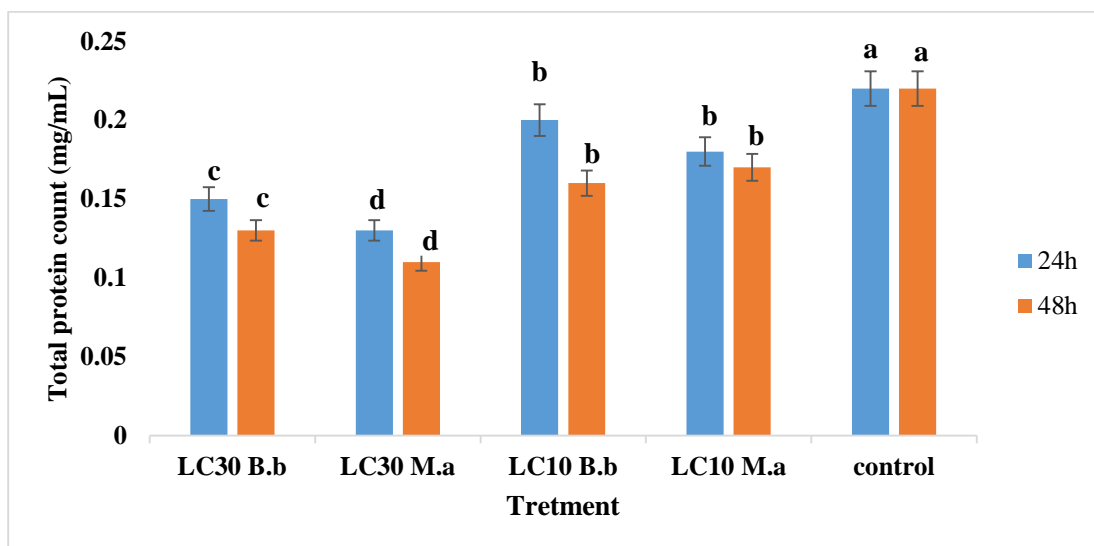
288
 289 **Table 6.** The effect of entomopathogenic fungi on plasmatocytes changes in the third instar larvae of *Helicoverpa*
 290 *armigera*.

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 <i>B. bassiana</i>	6.25±0.47 b	8.25±0.75 c	11.75±0.85 c	10±0.4 d
LC ₃₀ <i>M. anisopliae</i>	6.25±0.47 b	7.5±0.64 bc	10.25±0.62 bc	8.5±0.64 cd
LC ₁₀ <i>B. bassiana</i>	5±0.4 ab	5.75±0.25 bc	9.5±0.64 b	7.5±0.64 bc
LC ₁₀ <i>M. anisopliae</i>	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

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

292
 293 **Estimation of Protein Concentration**

294 The results showed that the injection of LC₁₀ and LC₃₀ concentrations of both fungi decreased
 295 the hemolymph protein concentration (Figure 3). 24 and 48 hours post-injection, larvae treated
 296 with LC₃₀ *M. anisopliae* and LC₁₀ *B. bassiana* had the lowest and highest protein
 297 concentrations, respectively, compared to the control.



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

301
 302 **Discussion**

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

310 mortality on larvae after five days. In our study, eight days after treatment, the lowest mortality
311 was observed at the concentration of 0.18×10^6 spore ml^{-1} . Petlamul *et al.* (2019) evaluated the
312 change in the activity of some enzymes and reported that the PO activity and glutathione-S-
313 transferase was increased immediately after infection and decreased after 48 hours. In our
314 study, PO activity was increased in the treatments compared to the control 24 and 48 h after
315 infection. **Insects increase the activity of PO enzyme after being attacked by pathogens to**
316 **enhance their defense mechanisms against infections (Duffield *et al.*, 2023).** Bali and Kaur
317 (2013) showed that PO activity in 3rd, 4th and 5th instar larvae of *Spodoptera litura* was affected
318 when exposed *B. bassiana*. The activity of PO was increased in the 3rd and 4th instar larvae. No
319 significant effect was observed on 5th instar larvae 24 hours after treatment.

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

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

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

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

350 In previous studies, various types of hemocyte have been identified in the hemolymph of the
351 *H. armigera*, including prohemocytes, plasmatocytes, granulocytes, spherulocytes,
352 oenocytoids, and coagulocytes (Essawy *et al*, 1985; Gujar and Kalia, 2005). In a study by Kalia
353 *et al* (2001), six types of hemocyte were identified in the hemolymph of cotton bollworms:
354 prohemocytes, plasmatocytes, granulocytes, oenocytoids, spherulocytes, and adipohemocytes.
355 Five types of hemocyte were identified in our research. In this study, the highest THC was
356 recorded 9 hours after infection by entomopathogenic fungi. The results showed that the
357 increase in hemocyte was directly related to the concentration of spores in the hemolymph. The
358 highest THC was observed in the LC₃₀ treatment of both pathogens. However, 12 hours after
359 infection, the number of hemocyte showed a decreasing trend in the fungi treatments, although
360 it was still significantly different from the control group.

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

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

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

389

390 **Acknowledgements**

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

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

531 اکرم حاتمی، رضا فرشباغ پورآباد، موسی صابر، و روح الله متفکر آزاد

532 **چکیده**

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