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RESEARCH REPORT

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

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Effects of *Beauveria bassiana* (Bals.-Criv.) spores on immune functions of *Glyphodes pyloalis* Walker larvae were studied. Total and differential hemocyte counts revealed that infection by *B. bassiana* caused a dramatic change in hemocyte number. *In vivo* and *in vitro* studies demonstrated that increase in time exposure to fungal pathogen resulted in elevated phagocytic activity. Nodules were formed in response to spore injection and their numbers were maximal 6 h post injection. The phenoloxidase (PO) activity in treated larvae changed significantly 3 and 6 h after spore injection compared with the control larvae. The reduction in PO activity was observed 12 and 24 h post injection. Effect of two developmental hormones, juvenile hormone (JH) and ecdysone on cellular immune response of *G. pyloalis* were also evaluated. Larval treatment with JH prior to *B. bassiana* spore injection reduced nodulation while ecdysone enhanced it. These results demonstrated that ecdysone and JH play an important regulatory role in the immune response in the studied insect.

Key Words: hemocyte; nodulation; juvenile hormone (JH); ecdysone; cellular immunity

Introduction

Entomopathogens are important regulatory factors of insect populations. At present, several species of entomopathogenic fungi are used as biocontrol agents in insect pests (Bogus *et al.,* 2007). The entomopathogenic fungus, *Beauveria bassiana* (Bals.-Criv.) shows great potential for the control of a broad range of insect species. This fungus has been developed for use as a biological pesticide (Ansari and Butt, 2012). The process of infection begins with attachment of the conidia to the cuticle. The fungus then germinates, grows and penetrates the integument. In addition to being able to reach the cuticle barrier, this pathogen possesses the ability to replicate in the insect hemocel (Xiong *et al.,* 2013). Upon entering the hemocel, fungal cells interact with insect hemocytes. They then, induce peptides and proteins that mediate humoral immunity (Borges *et al.,* 2008).

Insects defend themselves from infection by a variety of potential pathogens including; bacteria, fungi and parasites in natural habitats. They have therefore evolved efficient host-defense mechanisms to survive (Yamauchi, 2001). The immunity system in insects consists of cellular and humoral defense responses. Humoral defenses refer to antimicrobial peptides, cell adhesion molecules, lysozyme, lectins, and the prophenoloxidase (proPO) (Hoffmann, 2003; Kanost *et al.,* 2004). Cellular defenses include responses such as phagocytosis, encapsulation, and clotting that with hemocytes acting as important mediators of such processes (Lavine and Strand, 2002). Several morphologically distinct hemocyte cell types work together in the immune reactions. Cellular reaction include phagocytosis in which individual hemocyte ingest large particles that enter the hemocel from the environment. This process is essential for host defense in higher eukaryotes against infectious microorganisms and for the elimination of apoptic cells generated during development. This is regarded as the main cellular reaction of innate immunity in vertebrates and invertebrates (Borges *et al.,* 2008). Foreign particles are identified by phagocytic cells via recognition by a series of receptors on cell membranes that bind to pathogen-associated molecules (Rosales, 2011). Two types of hemocytes involved in phgocytosis are the granulocytes and the plasmatocytes. However,

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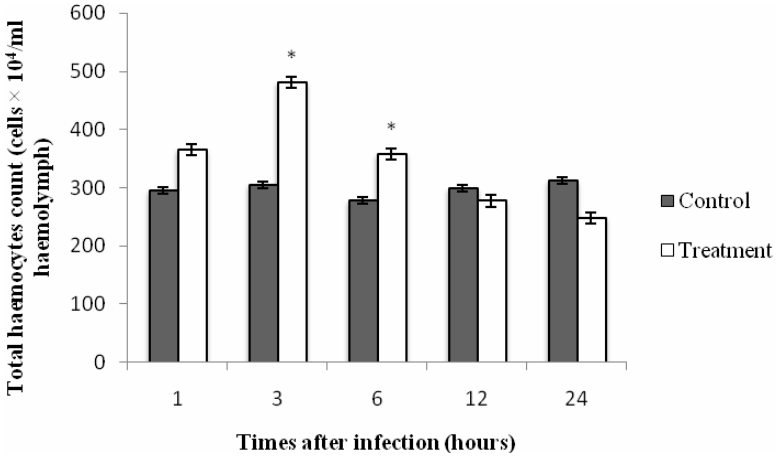

Fig. 1 Total hemocyte count (cells ×10^4/ml) of *G. pyloalis* infected with *B. bassiana* spores. *Means ± SE followed by the asterisk indicate significant differences versus control ($p < 0.05$) according to the Tukey's test.

their contribution in this process varies between insect species (Moushumi *et al.,* 2008). After entering the hemocel, micro-aggregations of hemocytes (granulocytes and plasmatocytes) are initiated on microorganisms. This process is initiated by changing of circulating hemocytes from non-adhesive to adhesive cells that are able to bind to microorganisms (Lavine and Strand, 2002). These micro-aggregation will eventually lead to the formation of nodules. In the later stages of nodule formation, melanization takes place within the nodules. Finally, foreign particles almost die. Several factors such as asphyxiation, production of toxic quinones or hydroquinones via the proPO cascade and antibacterial peptides have been proposed to function as killing agents (Nappi and Christensen, 2005).

Phenoloxidases (POs) are vital enzymes involved in a number of crucial processes, such as defense, wound healing, coagulation, sclerotization, and melanization (Bogus *et al.,* 2007). POs are present as inactive precursor, the proPO in insect hemolymph and are activated in response to wounding or infection as a part of the innate immune response (Cerenius *et al.,* 2008). These enzymes are similar to mammalian tyrosinases in their ability to use reactive sites containing copper atoms to catalyze two types of reactions that require molecular oxygen as a substrate. The cytotoxic quinones that are produced during oxidation are placed over the surface of large foreign materials in order to form melanized layers, which help to kill the encapsulated fungi, bacteria or parasites (Ling *et al.,* 2005).

Table 1 Number of hemocyte types from control and treated larvae of *G. pyloalis* with *B.bassiana* at different times after the immune challenge.

	Control					**Treatment**				
	1 h	**3h**	**6h**	**12 h**	**24 h**	**1 h**	**3 h**	**6 h**	**12 h**	**24 h**
Pr	20.28±2.02	17±2.9	18±1.89	18.6±2.03	15.6±1.69	20.2±1.76	17.4±1.74	19.6±1.44	15±1.49	18±1.89
Pl	107.6±3.36	124.6±3.59	112.4±2.85	119±2.07	123±3.8	185.2±3.64*	214±1.81*	183±3.22*	99.8±4.41*	86±4.84*
Gr	52.4±2.4	57.2±1.06	65±3.48	62±2.47	68.2±2.26	67±3.61*	110±3.96*	72±3.02*	49±4.13*	41±3.42*
Sp	7.2±0.91	8.6±1.28	11.2±1.28	10±1.25	9±1.1	7.6±1.06	9±1.31	9±1.0*	8.2±1.21*	8.2±0.91
Oe	10.6±1.39	9.8±2.12	10±1.23	9.8±1.21	8.6±0.94	13.4±1.29*	12±1.45	11.2±1.43	13±1.49*	9.6±1.23

Pr= Prohemocyte; Pl= Plasmatocyte; Gr= Granulocyte; Sp= Spherulocyte; Oe= Oenocytoid

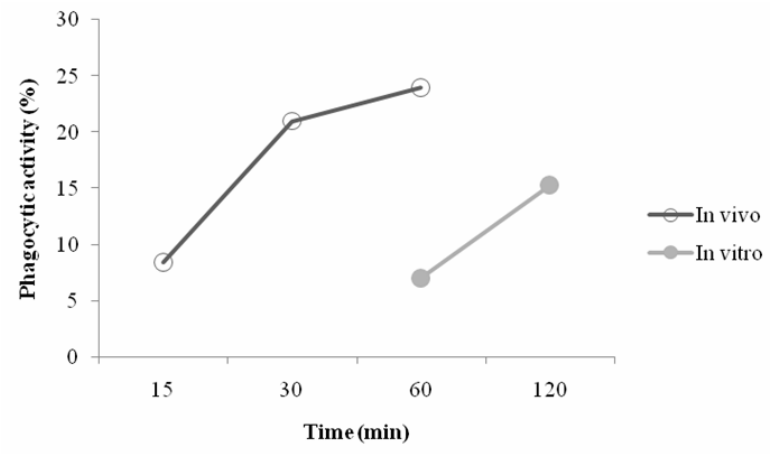

Fig. 2 *In vivo* and *in vitro* phagocytosis of *B. bassiana* spores by plasmatocytes and granulocytes.

Endocrine factors are involved in the immune system and mediate immune signals. The eicosanoids (Stanley, 2000), biogenic amines (Baines *et al.,* 1992) adipokinetic hormone (Goldsworthy *et al.,* 2003), juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Franssens *et al.,* 2006) change insect immune response to pathogenic agents. Inhibition of hemocyte encapsulation response was observed in *Tenebrio molitor* after injection of JH (Rantala *et al.,* 2003). The 20E promotes nodule formation of *Neobelliera bullata* in response to injection of laminarin, the components of bacterial cell wall (Franssens *et al.,* 2006).

The lesser mulberry pyralid, *Glyphodes pyloalis* (Lepidoptera: Pyralidae) is a specialist insect on mulberry, and is widely distributed throughout Asia and the northern province of Iran. This pest has caused severe damage to mulberry plantations in northern Iran and has posed a serious concern to silkworm growers. Fifth instar larvae feed on the whole leaf until only the ribs remain (Khosravi and Jalali Sendi, 2010).

Populations of most pests are usually regulated by density dependent factors involving pathogens and parasites. Thus, understanding the interactions of pest with their pathogens and parasites and hence, the cellular defense responses are needed for developing best methods of pests' control. Hemocytes perform certain vital activities in insects, and thus hematological studies are fundamental in the field of insect physiology (El-Aziz and Awad, 2010). An important necessity in the study of the fungal pathogen host interaction is the ability to detect mechanisms that are used by pathogens to overcome the insect's immune defense system.

Table 2 THC and number of plasmatocytes and granulocytes (×10^4 cell/mL) of *G. pyloalis* larvae at 3, 6 and 12 h after JH treatment prior to *B. bassiana* spore injection.

	THC			Number of plasmatocytes			Number of granulocytes		
	3 h	**6 h**	**12 h**	**3 h**	**6 h**	**12 h**	**3 h**	**6 h**	**12 h**
K	347.67 ±4.21a	337.67± 4.78a	292.33± 3.53a	215± 6.13a	241.33± 3.01a	239.66± 2.22a	85.66± 2.74a	87.3±3. 16a	84.00± 1.62a
A	323.67 ±3.6a	278.00± 4.6b	251.67± 3.64b	220±2. 08a	181.00± 3.04c	166.00± 1.58c	68.33± 2.24b	69.66± 2.42b	69.33± 2.21b
B	332.47 ±3.09a	287.33± 4.3b	256.33± 3.53b	224±2. 95a	193.00± 2.56bc	173.33± 2.12c	69.66± 2.24b	64.3± 1.74b	67.00± 2.46b
C	330.00 ±3.92a	307.00± 2.79ab	283.33± 3.96ab	213.6± 1.87a	191.33± 2.42bc	213.33± 2.58ab	73.00± 2.64ab	75.00± 1.89ab	66.4±1. 44b
D	327.43 ±2.38a	309.67± 3.53ab	276.33± 2.53ab	224.5± 2.88a	201.00± 3.14b	198.00± 3.08bc	75.65± 1.87ab	74.66± 2.85b	74.32± 2.17ab

K = Control; A = JH 0.5 mg/mL; B = JH 0.25 mg/mL, C = JH 0.125 mg/mL; D = JH 0.062 mg/mL. Means ± SE within the same column followed by the same letter are not significantly different ($p \leq 0.05$ Tukey test).

This study was undertaken to investigate the effects of *B. bassiana* isolate Fashand on cellular immune responses and the PO activity of *G. pyloalis*. Secondly, in order to understand the role of insect hormones in immune responses, the effects of JH and ecdysone, two key insect hormones on immune reactions of this pest were evaluated.

Materials and methods

Insects rearing

Larvae of *G. pyloalis* were collected from mulberry orchards (Kenmuchi Var.) in Rasht (37°16′51″N 49°34′59″E), north of Iran. They were maintained in the laboratory in a rearing chamber of constant temperature (25 ± 1 ˚C), relative humidity (75 ± 5 %) and photoperiod (16:8 h light:dark). Larvae were placed in plastic jars 10×20 cm and were daily provided with fresh mulberry leaves (Kenmuchi Var.). On adult emergence, they were transferred to 18×7 cm transparent jars and were provided with fresh leaves for egg laying and cotton wool soaked in 10 % honey for feeding.

Beauveria bassiana culture

B. bassiana isolate Fashand was grown in sterile petri dishes containing potato dextrose agar (PDA) and were incubated at 25 ± 1 ˚C in complete darkness. Spores were harvested from PDA plates with a sterile scalpel after 14 days. The final concentration was adjusted to 1×10^5 spores/ml using a hemocytometer in distilled water containing 0.01 % of Tween 20.

Injection of insects with spores

Fifth instar larvae of *G. pyloalis* were immobilized on ice for 5 min and were surface sterilized with 70 % ethanol. This experiment was replicated three times and ten insects were used in each replicate. After injection with 1×10^5 spores/ml (2 µL) by a 10 µL Hamilton syringe, the larvae were transferred to rearing jars provided with fresh mulberry leaves, to follow the course of the assay for further observation. The control larvae were injected with distilled water containing 0.01 % of Tween 20 (2 µL) alone.

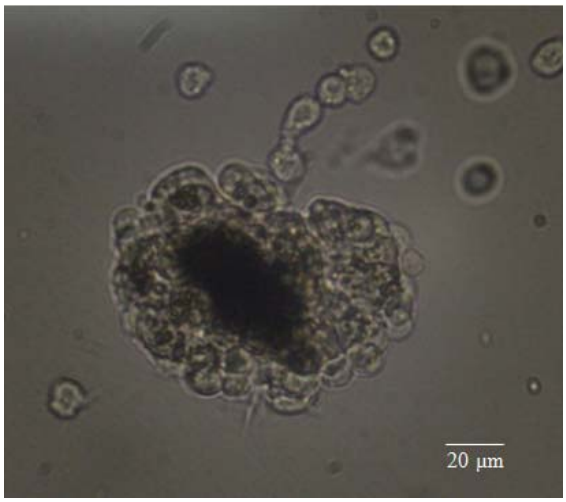

Fig. 3 Induction of nodule formation in *G. pyloalis* larvae with 2 µL*B. bassiana* spores injection.

Effect of fungal spores of B. bassiana on hemocyte number

To determine, whether injection of spores of *B. bassiana* could cause any changes in the levels of total and differential hemocyte numbers, the fifth instar larvae were injected with 2 µL of 1×10^5 spores/mL concentration between the second and third prolegs. Similarly, the controls were injected with 2 µL of sterile distilled water containing 0.01 %

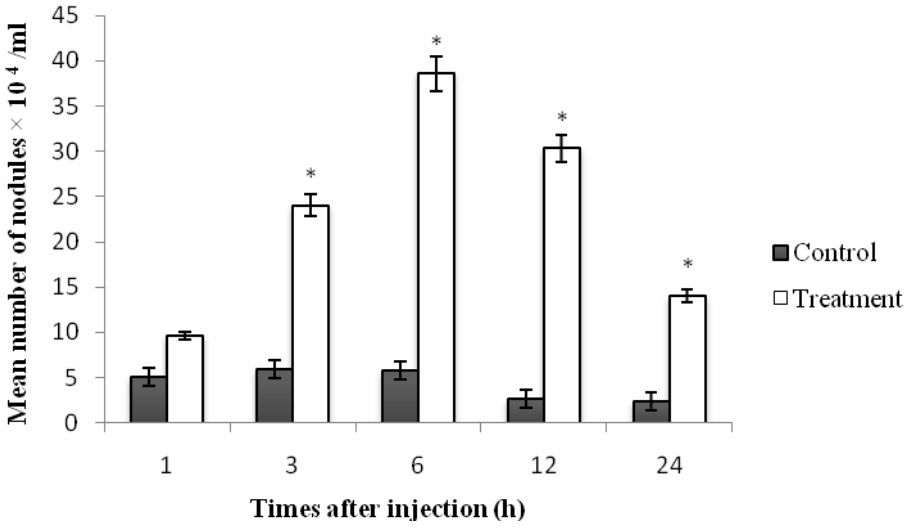

Fig. 4 Effect of *B. bassiana* spores on nodule formation in fifth instar larvae of *G. pylalis*. *Means ± SE followed by the asterisk indicate significant differences versus control ($p < 0.05$) according to the Tukey's test.

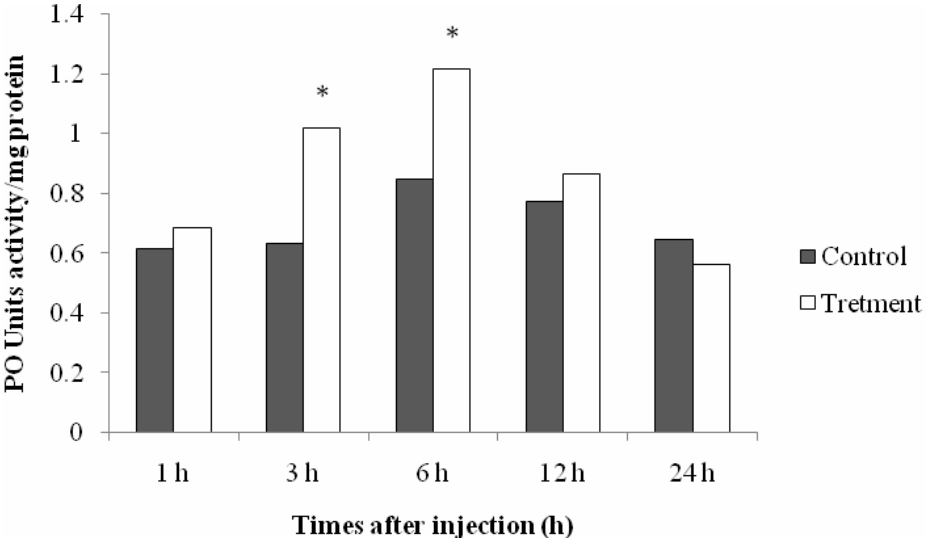

Fig. 5 Effect of *B. bassiana* spores on phenoloxidase (PO) activity in fifth instar larvae of *G. pylalis*.
*Means ± SE followed by the asterisk indicate significant differences versus control ($p < 0.05$) according to the Tukey's test

of Tween 20. Hemolymph was collected by cutting one of the prologs 1, 3, 6, 12, and 24 h after injection from chilled, surface sterilized (70 % ethanol) larvae. This experiment was replicated three times and ten insects were used in each replicate. Samples of hemolymph from each larva were diluted 5-fold with a cold anticoagulant buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA and 0.041 M citric acid, pH 4.5). Then, the total and differential hemocyte numbers were counted on an improved Neubauer hemocytometer for each treatment (El-Aziz and Awad, 2010).

Labeling of B. bassiana spores

The spores of *B. bassiana* for labeling were obtained from the 10 - 14 day culture on PDA medium. The spores were re-suspended in 10 mL of phosphate buffered saline (PBS) (0.13 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4, autoclaved), were then washed and re-suspended in a sterile CO_3-HCO_3 buffer at pH 9.4 (9.5 mL 0.2 M Na_2CO_3 was mixed with 41.5 ml 0.2 M $NaHCO_3$). The solution was made up to 200 ml and was labeled by mixing this solution with 1mg of FITC (Fluorescein Isothiocyanate, Sigma) on a shaker for 30 min at room temperature in complete darkness (Rohloff *et al.,* 1994).The spores were rinsed by phosphate buffered saline four times, pelletized, and the pellets from the last wash were re-suspended in 1 mL of Grace's insect medium (GIM, Gibco) and stored at -20 ˚C until needed.

Phagocytosis assay

The phagocytic activity of hemocytes was assessed using the FITC-labeled *B. bassiana* spores. Larvae were injected with 2 µL of 1×10^5 FITC- labeled *B. bassiana* spores/mL and phagocytic activity of hemocytes was investigated 15, 30, and 60 min post injection. This experiment was replicated three times and ten insects were used in each replicate. The body surface of each larva was sterilized with 70 % ethanol before extracting the blood. The larvae were bled by cutting one of the prolegs. The hemolymph was then collected into ice-cold anticoagulant solution and poured over microscopic slide. The slide was incubated in a moist dark chamber at room temperature for 5min with 10 µL of trypan blue solution (2 mg/mL) in order to quench spores that were not ingested.

For the *in vitro* phagocytosis assays, 10 µL of FITC-labeled *B. bassiana* spores were mixed with 10 µL of freshly collected hemolymph on a microscopic slide and were then incubated in a moist dark chamber at room temperature for 60 and 120 min. Then, 10 µL of trypan blue solution was added and the mixture was incubated for another 5 min.

In both the tests the phagocytic activity was determined by counting hemocytes with or without ingested spores under a fluorescence microscope (Leica, Wetzlar, Germany). Ten photo-frames per microscope slide were counted and the average was calculated. Each experiment was repeated for 3 times (Tseng *et al.,* 2008). Phagocytosic activity was calculated as the number of phagocytosing cells×(number of total cells)$^{-1}$×100.

Effect of fungal spore on nodulation

Injections were carried out as mentioned above. The number of nodules formed at 1, 3, 6, 12, and 24 h post injection were determined. Hemolymph was collected from each larva, then samples in 5 replicates were poured into a hemocytometer, and the number of nodules was counted (Franssens *et al.,* 2006).

Assay for PO activity

PO activity was measured according to the procedure of Catalan *et al.* (2012) with slight

modification. In order to test the effect of *B. bassiana* spores on the PO system in *G. pyloalis* larvae, 10 µL of hemolymph was diluted with 90 µL of ice-cold sterile phosphate buffered saline and then were vortexed. Samples were frozen at -20 ˚C for 48 h. For assay of PO activity, L-DOPA was used as a substrate (Wilson *et al.,* 2001; Cotter *et al.,* 2004). Then samples were centrifuged at 5,000g at 4 ˚C for 5 min. Fifty microliters of hemolymph-buffer supernatant was mixed with 150 µL of L-DOPA (10 mM). PO activity was measured (in 3 replicates) at 490 nm for 30 min in 5 min intervals using a microplate reader (Awareness Technology Inc, Florida, USA).

Protein determination

The method of Bradford (1976) was used for determining total protein, using bovine serum albumin (Bio-Rad, Munchen, Germany) as the standard.

Effect of ecdysone and Juvenile hormone (JH) on immune responses

Ecdysone was dissolved in Ringer's solution (0.123 M NaCl, 1.53 M $CaCl_2$, 4.96 M KCl, pH 7.4) at a concentration of 5 mg/mL. Initially, preliminary tests were performed to find the effective dose ranges of ecdysone and JH on the development of *G. pyloalis*. Then the stock solution of ecdysone was diluted with ringer to a concentration of 0.5, 0.25, 0.125, and 0.062 mg/mL. JH was dissolved in acetone at 5, 2.5, 1.25 and 0.625 mg/mL and 2 µL of this solution was topically applied onto the metathoracic tergum of each larva. Four hours after topical application of JH on metathoracic tergum or injection of Ecdysone, a suspension of *B. bassiana* spores (1×10^5 spores/mL) was prepared and 2 µL of which was injected to the larva by a Hamilton syringe. Then, after 3, 6, and 12 h total and differentiated hemocyte number and nodules were counted. Control specimens were either first topically treated with 2µL of acetone or were first injected with Ringer's solution and then with *B. bassiana* spores. Three replicates were used for each concentration (N = 30) and totally 120 insects were used for immunological assays.

Statistical analysis

All data obtained from the experiments were subjected to analysis of variance (ANOVA) ($p < 0.05$). Means were compared by Tukey's studentized range test, accepting significant differences at $p \leq 0.05$ (SAS Institute, 1997).

Results

Effect of B. bassiana spores on hemocyte number, phagocytosis and nodulation

Total number of circulating hemocytes in lesser mulberry pyralid, *G. pyloalis* fifth instar larvae exhibited major changes after *B. bassiana* spore injection (Fig. 1). A significant increase in total hemocyte count (THC) was recorded 3 and 6 h post injection (365 and 481×10^4 cell/mL respectively), while in control THC was recorded only 295 and 305×10^4 cell/mL, respectively ($p \leq 0.05$).

As shown in Table 1, significant changes in the profile of five hemocyte types were observed in various intervals post-injection of *B. bassiana* spore compared with the control. The number of plasmatocytes and granulocytes increased 1, 3, and 6 h post- injection, and then decreased after 12 and 24 h. Fungal infection generally increases the number of plasmatocytes in first interval after the inoculation. The data revealed that the numbers of oenocytoids were increased significantly after 1 and 12 h pos-injection compared with the control. The number of prohemocytes did not change significantly but the number of spherulocytes decreased 6 and 12 h post injection of *B. bassiana* spores.

Hemocytes of *G. pyloalis* showed a basic phagocytosis activity against *B. bassiana* spores. Results of this study showed that plasmatocytes and granulocytes of *G. pyloalis* have an important role in phagocytosis of foreign particles. The most phagocytic activity was observed 30 and 60 min after injection of spores. The phagocytic potential of *G. pyloalis* was higher *in vivo* than *in vitro* (Fig. 2).

Nodule formation (Fig. 3) in *G. pyloalis* larvae was significant after injection of *B. bassiana* spores. Most of the nodules occurred 3, 6 and 12 h after inoculation of fungi. The highest number of nodules could be observed 6 h post injection, and then decreased 24 h post-injection (Fig. 4).

Effect of B. bassiana spores on phenoloxidase activity

When fifth instar larvae of *G. pyloalis* were injected with *B. bassiana* spores (1×10^5), the PO system was activated during intervals after inoculation (Fig. 5). The highest PO activity was observed 3 and 6 h after the injection, and then decreasing after 12 and 24 h but not significant statistically.

Effect of exogenous ecdysone and JH on immune responses in G. pyloalis

Quantitative analysis of THC of insects treated with exogenous JH is shown in Table 2. By increasing the concentration of exogenous JH from 0.625 to 5 mg/mL a dose-dependent decrease in total number of hemocytes was observed compared with the control. Plasmatocyte numbers sharply decreased along with increase in the concentrations. The number of granulocytes decreased at higher concentrations compared with the control.

The changes of total hemocyte count in the fifth instar *G. pyloalis* larvae were affected by injection of exogenous ecdysone. As shown in Table 3, total hemocyte count significantly increased with time and concentration. Higher concentrations of exogenous ecdysone seemed to play a strong facilitating role in promoting the increase in granulocyte numbers.

Treatment of larvae with exogenous JH significantly inhibited the nodule formation in larvae injected with *B. bassiana* spores (Fig. 6A). While, increasing concentrations of exogenous ecdysone (0.5 and 0.25 mg/mL), enhanced nodulation 3 and 6 h after *B. bassiana* injection (Fig. 6B).

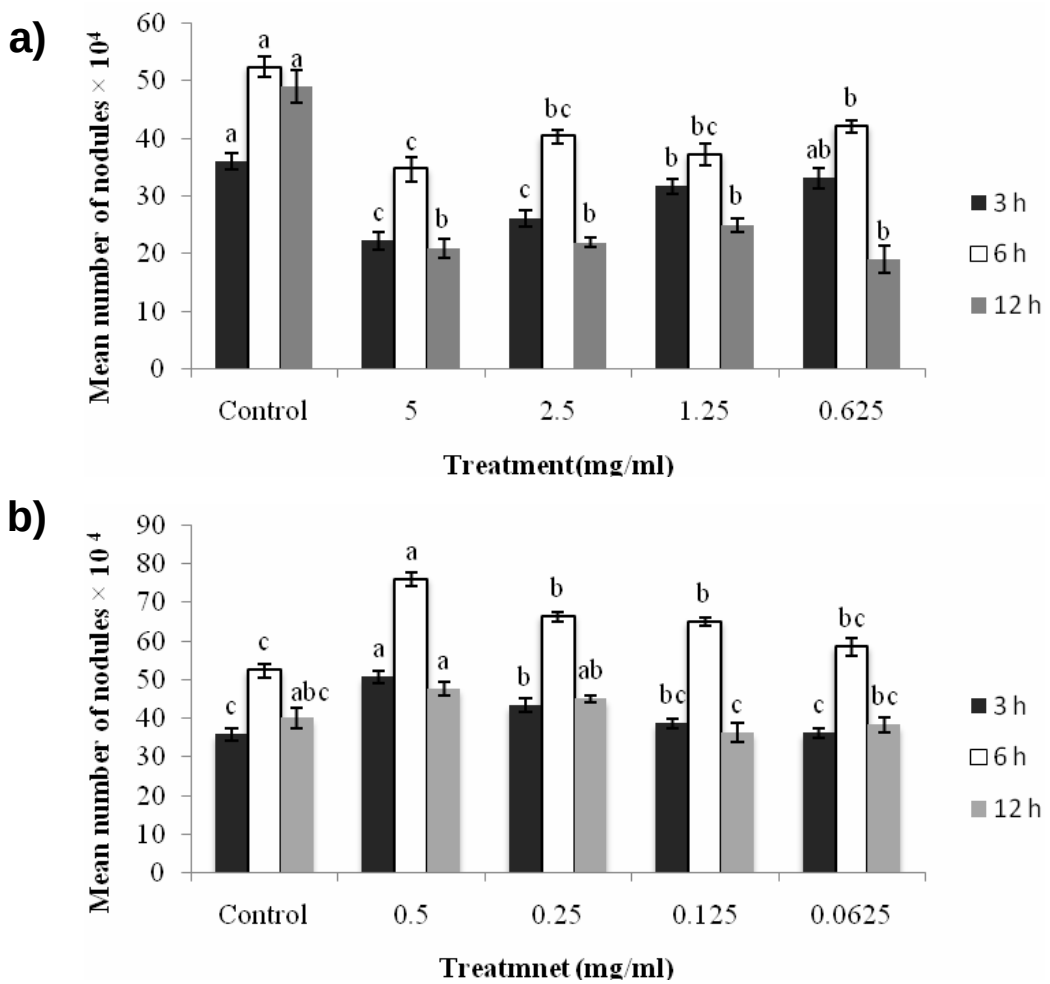

Fig. 6 Influence of different concentrations of JH (A) and ecdysone (B) on nodule formation of *G. pyloalis* larvae at 3, 6 and 12 h after injection with *B. bassiana* spores. Data of each treatment at any time were compared with control at the same time.

Discussion

To understand the role of hemocytes and their involvement in the defense reactions of lesser mulberry pyralid larvae a pathogenic agent was used. It was found that, total hemocyte count (THC) first increased during infection, then declined 12 and 24 h post-injection.

As the infection progressed the THC and more specifically the granulocyte number were reduced. Similar reduction in circulating hemocytes was reported for *Spodoptera exigua* infected by *B. bassiana* (Hung and Boucias, 1992). Previous studies by other researchers showed a major effect of pathogenesis on THC in insects (Moushumi *et al.,* 2008; Ajamhassani *et al.,* 2013). Bandani (2008) reported no significant reduction in the total hemocyte count (THC) when *Galleria mellonella* larvae were injected with *Tolypocladium cylindrosporum* spores until 24 hours post injection. However, THC was suppressed 48 h post-treatment of larvae with spores. Meshrif *et al.* (2011) also indicated that population of *S. littoralis* hemocytes 48 h post injection of *B. bassiana* spores were significantly lower than the control, but 72 h post injection they were increased. The decline in THC in the later stages of infection could partly be attributed to the hemocyte aggregation (nodule formation), induced by soluble wall components released from circulating fungus (Gillespie *et al.,* 2000). Furthermore, cytotoxic fungal metabolites may play a key role in the reduction of hemocyte numbers.

The differential hemocyte count (DHC) showed an initial increase in plasmatocytes (PLs) and granulocytes (GRs) during infection, followed by a decline in these cell numbers. This result was in accordance to other reports (Gunnarsson, 1987; Pech and Strand, 1995; Gillespie *et al.,* 2000; Meshrif *et al.,* 2011). The total granulocyte and plasmotocyte numbers also increased 12 h after treatment with *Metarhizium anisopliae* in *Oxya japonica* (Anggraeni and Putra, 2011). PLs and GRs are known to give out cytoplasmic processes in retaliation to any invading foreign material (Sharma *et al.,* 2008).

Table 3 THC and number of plasmatocytes and granulocytes (×10^4 cell/mL) of *G. pyloalis* larvae at 3, 6 and 12 h after ecdysone treatment prior to *B. bassiana* spore injection

	THC			Number of plasmatocytes			Number of granulocytes		
	3 h	**6 h**	**12 h**	**3 h**	**6 h**	**12 h**	**3 h**	**6 h**	**12 h**
K	341.33± 4.26a	326.00± 3.88c	292.23± 3.53c	215.00± 4.13ab	241.3± 3.01b	239.66± 2.22ab	79.00± 2.74b	87.26± 3.16c	84± 1.62c
A	373.64± 3.17a	423.0± 3.18a	386.00± 3.43a	257.22± 3.53a	304.43± 2.60a	254.33± 4.26a	108.00± 3.08a	143.66± 3.16a	110.4± 3.6b
B	362± 4.28a	392.65± 3.45ab	354.3± 4.32ab	232± 2.56ab	270 ±3.48b	240± 3.47ab	80.3± 2.76b	131± 2.92ab	124.33± 1.72a
C	358.33± 6.36a	363.2± 6.62bc	323.67± 6.23bc	221± 5.46ab	236.12± 4.98b	259± 3.48a	106± 3.6a	119.42± 4.09b	120± 1.44ab
D	334.43± 2.73a	337.5± 3.17bc	329.± 2.49bc	207.35± 4.93b	243.67± 5.36b	221.27± 3.08b	91.2± 3.6ab	110.1± 3.53b	123.3± 2.24a

K = Control; A = ecdysone 0.5 mg/mL; B = ecdysone 0.25 mg/mL, C = ecdysone 0.125 mg/mL; D = ecdysone 0.062 mg/mL. Means ± SE within the same column followed by the same letter are not significantly different ($p \leq 0.05$ Tukey test).

In the current study we have demonstrated that oenocytoides' (OE) number changed in response to *B. bassiana* spores. Changes in the numbers of OEs following fungal injection may be attributed to the beginning of humoral activity or the active transformation of GRs into SPs and OEs as suggested by Gupta (1985).

The decrease of PLs and GRs can be attributed principally to their involvement in nodule formation and partially to programmed cell death induced by toxins secreted by the growing fungi. The decline in GRs observed may be due to their involvement in the latter stages of nodule formation, as has been reported in other insects (Gotz and Boman, 1985; Pech and Strand, 1995; Gillespie *et al.,* 1997). A significant increase in the percentage of GRs was observed in latex bead-treated insects 60 and 120 min after the inoculation. The percentage of oenocytoides significantly varied in response to *Staphylococcus aureus* infection, with an initial increase at 30 min followed by accentuated decrease 60 and 120 min post-inoculation (Borges *et al.,* 2008). In the process of phagocytosis cells recognize, bind and ingest relatively large elements and protect insects. Several authors have indicated that PLs are the major cell type involved in phagocytosis (Ratcliffe *et al.,* 1985; Anggraeni and Ratcliffe, 1991; Rohloff *et al.,* 1994). Immune reactions of *G. pyloalis* against *B. bassiana* showed that PLs and GRs are the main factors in phagocytosis and nodulation. In this study, most of phagocytotic activity occurred 30 and 60 min after injection.

This study showed that nodules are formed in response to injection of *B. bassiana* spores. The maximal number of nodules were observed 3, 6 and 12 h post-injection, but declined later. Similar results were recorded by other researchers (Gillespie *et al.,* 2000; Meshrif *et al.,* 2011; Ajamhassani *et al.,* 2013). It is generally agreed that the formation of nodules is initiated by degranulation of GRs, and the contents released act as an opsonin in the employment of other hemocytes, mainly PLs (Ratcliffe *et al.,* 1985). Contact of GRs with the fungus, or the release of β-1, 3 glucan of other soluble material from the fungus could be the cause of this initial degranulation (Gillespie *et al.,* 2000). The subsequent decline in nodule numbers may be due to their running out of circulation and attaching to the body wall when they attain a stable size (Brookman *et al.,* 1989). It is likely that the immunosuppressive effects of fungal metabolites might have a role in their decline. The dark color of the nodules demonstrates the synthesis of melanin and at least a localized activation of proPO, which is indicative of their formation by hemocytes (Lavin and Strand, 2002).

PO is responsible for the activation of melanogenesis in invertebrates. The main role of PO in melanogenesis is to convert phenols to quinones which subsequently polymerize to form melanin (González-Santoyo and Córdoba-Aguilar, 2011). Furthermore, recent research has documented PO as an important tool used against several pathogens (Cerenius and Söderhäll, 2004). Wounding or infection activates PO system as part of immune response (Kanost and Gorman, 2008). In the present study we observed high level of PO activity in hemolymph of larvae injected with *B. bassiana* spores 3, 6 and 12 h post-injection. Gillespie *et al.* (2000) demonstrated that a topical application of conidia from *M. anisopliae* var. *acridum* led to significant elevation of proPO in the hemolymph.

We investigated the effect of different concentrations of JH and ecdysone on immune parameters including total hemocyte counts and nodule formation. We found that high concentrations of JH reduced total hemocyte number. Also both plasmatocyte and granulocyte numbers sharply

decreased in a time and concentration-dependent manner. Zibaee *et al.* (2012) demonstrated that Pyriproxyfen (pyridine-based pesticide with JH mimicking activity) caused significant reduction in THC, plasmatocyte and granulocyte population in *Eurygaster integriceps* adults. Kim *et al*. (2008) showed a dose response relationship for 20E on the number of plasmatocytes in *Spodoptera exigua*.

Treatment of *G. pyloalis* larvae with JH caused reduction in the ability of the larvae to form nodules in response to injection of *B. bassiana*. Similar results have been reported by Rantala *et al.* (2003) and Zibaee *et al.* (2012).

We have also assessed the influence of ecdysone on total hemocyte count and nodulation. It was observed that ecdysone stimulated nodule formation in a dose dependent manner. When larvae of *N. bullata* were treated with 20E prior to laminarin (a storage glucan) injection increased the nodulation response in a dose-dependent manner. Contrary to ecdysone treated larvae with JH or juvenile hormone analogs (JHA), fenoxycarb and pyriproxyfen, significantly reduced the formation of nodules in response to laminarin (Franssens *et al.,* 2006). The phagocytic activity of plasmatocytes in larvae of *N. bullata* was enhanced after 20E injection (Lanot *et al.,* 2001). It was also shown that 20E signaling was needed for *Drosophila* lymph gland growth and hematopoiesis, both of which are required for pathogen encapsulation (Sorrentino *et al.,* 2002). In the grey flesh fly larvae *N. bullata*, 20E promotes cell-mediated nodulation response (Franssens *et al.,* 2006).

In the tobacco hornworm *Manduca sexta* JH acts as inhibitor for PO synthesis and thus cuticular melanization did not occur (Hiruma and Riddiford, 1988). Similarly in the mealworm beetle *T. molitor*, JH reduced immune parameters such as PO levels and encapsulation (Rolff and Siva-Jothy, 2002; Rantala *et al.,* 2003).

From these results, it is well established that 20E positively regulate the innate immune system of insects, while JH works as an immuno-suppressor (Flatt *et al.,* 2005). Flatt and Kawecki (2007) showed that JH and 20E have hostile effects on the induction of antimicrobial peptide genes in fruit fly, *Drosophila melanogaster*.

The present study demonstrated that the entomopathogenic fungus, *B. bassiana* has properties that allow for its successful replication in lesser mulberry pyralid and strongly affect the immune responses of this pest. The understanding of interaction between entomopathogenic fungus and the insect is an important step in fungal disease propagation. Further, understanding fungal induced immune response and the identification of fungal virulence factors and their targets may provide significant methods for development of efficient mycoinsecticides in biological control of dangerous pests. JH and ecdysone have significant effect on immune response of *G. pyloalis* to *B. bassiana* spores. It can be stated that the results of this experiment suggests JH or its analogues as an immunosuppressive agent and may be used in a control program prior to use of fungal pathogens while, ecdyson facilitates effective immune reactions.

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