IMMUNOLIOGICAL RESPONSES OF *HYPHANTRIA CUNEA* (DRURY) (LEPIDOPTERA: ARCTIIDAE) TO ENTOMOPATHOGENIC FUNGI, *BEAUVERIA BASSIANA* (BALS.-CRIY) AND *ISARIA FARINOSAE* (HOLMSK.) FR.

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Abstract: Five morphological types of hemocytes were recognized in hemolymph of the 4th instar larvae of *Hyphantria cunea* (Drury). These hemocytes were: prohemocytes, plasmotocytes, granulocytes, oenocytoids, and spherulocytes. Tests were done on the effects of four isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criy) (Fashand, spt-22, Ir-K-40 and 566), one isolate of *Isaria farinosae* (Holmsk.) Fr. (1872c), and latex-beads on the cellular immune defense mechanism and Phenoloxidase (PO) activity of *H. cunea.* Observation showed that plasmotocytes and granulocytes engulfed fungal pathogens by phagocytosis. The most phagocytosis occurred 30 and 60 min after injection but nodulation occurred in 3 and 6 hours, in all treatments. The total hemocyte count (THC) and granulocyte, and plasmotocyte numbers increased after the injection of spores. Phenoloxidase activity was determined in the presence of L-DOPA (L-3,4-dihydroxyphenylalanine), as a substrate in intervals, after injection of fungal spores, and latex beads. These studies demonstrated that *B. bassiana* is a promising candidate for biological control of *H. cunea.*

Key words: *Beauveria bassiana*, hemocyte, *Hyphantria cunea,* immunity

INTRODUCTION

The fall web worm, *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) is a polyphagous insect. It is native to North America and later became an invasive alien species in some parts in Europe, for example in Italy, Austria, Spain and France (Montermini and Oliva 1984; Wittenberg 2005). In August 2002, specimens of *H. cunea* were found in Iran for the first time, in the Caspian forests near the city of Lasht-e-Nesha in the province of Guilan. In this area, populations of *H. cunea* have become widely established during the last few years and feed voraciously on a wide range of forest and fruit trees, shrubs, and herbaceous plants. About 600 plant species have now been identified as potential hosts (Rezaei *et al.* 2006). In Iran, the application of Dimilin and some environmentally safe materials including *Bacillus thuringiensis* var Kurstaki and Neem (*Azadirakhta indica* Juss.) extract on mulberry leaves have been tested (Yarmand *et al.* 2006). They have published the only known report about an entomopathogenic agent affecting the pest *H. cunea*. Like other entomopathogenic fungi, *Beauveria bassiana* (Bals.-Criy) possess the ability to produce infectious conidia. This conidia penetrates the insect's cuticle, indicating that, *B. bassiana* induces an appropriate mechanism to overcome the insect's cellular defense system (Abood *et al.* 2010). The

study on the interaction between entomopathogens and immune reaction is of immense importance and proves the ability of one to overcome the other. Hematological studies are an important field of insect physiology. It is hemocytes which perform certain vital activities (Nahla *et al.* 2010). Insect hemocytes are important mediators of cellular defense reactions (Hazarika and Gupta 1987; Kurtz and Sauer 2001). Hemocyte types and their specific responses during insect-pathogen interaction are good indicators of insect defense reactions (Da Silva *et al.* 2000; Gillespie *et al.* 2000). Several classes of hemocytes have been morphologically and functionally characterized, mainly in species such as *Diptera*, *Lepidoptera* and *Coleoptera* (Lavine and Strand 2002; Giulianini *et al.* 2003; Costa *et al.* 2005; Giglio *et al.* 2008). The most common hemocyte types are prohemocytes, plasmotocytes, granulocytes, and oenocytoids. These cells have been identified in a wide range of insects (Gupta 1985). Cellular immunity includes phagocytosis, encapsulation, and nodule formation (Lavine and Strand 2002). The first step of a cellular reaction is phagocytosis. During the phagocytic process, cells ingest large particles of fundamental importance to an insect's development and survival. Phagocytic cells recognize foreign particles through a series of receptors on cell membranes for pathogen-associated molecules.

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These receptors, in turn, initiate a series of signaling pathways that instruct the cell to ingest and eventually destroy the foreign particle. (Rosales 2011). During the next stage**,** micro-aggregates of granulocytes and plasmatocytes surround fungal pathogens to form nodules (Ratcliffe and Gagen 1976). Granulocytes and plasmatocytes react by altering their morphology. Some plasmatocytes are swollen; many have irregular shapes due to the rupturing of the plasma membrane causing cell contents to leak (Moushumi *et al*. 2008). If larger pathogens like parasitoids and nematodes invade the hemocel of an insect, encapsulation will take place. Finally, foreign particles are removed from hemolymph by the melanization process. (Moushumi *et al*. 2008). Antimicrobial peptides, cell adhesion molecules, lysozyme, lectins, and pro phenoloxidase systems all play a major role in humoral defense. Phenoloxidase is a key factor in immunity and plays a major role in coagulation, melanization, and wound healing (Soderhall and Cerenius 1993).

Butt *et al*. (2001) reported that fungus has great potential for use as a biological control agent. Due to the high level of damage caused by *H. cunea* in northern Iran and most countries in Europe and North America, biological control agents like pathogens can be regarded as an effective way of controlling this insect species. Application of entomopathogenic fungi is considered as a priority, as its use will facilitate a decrease in the harmful side effects of chemical pesticides. Therefore, this research was done to identify different types of hemocyte in *H. cunea,* and to study the effects of four isolates; *B. bassiana*, *Isaria farinose* (Holmsk.) Fr. 1832*,* and latex-beads on the cellular defense and phenoloxidase activity of these insects.

MATERIALS AND METHODS

Insect Rearing

Eggs of *H. cunea* were collected from the forests of Rasht in northern Iran, and reared on mulberry leaves (Kenmochi variety) in the laboratory, at 26±1°C, 85% relative humidity (RH) and 14:10 L:D. After hatching, larvae fed on leaves. Fourth instar larvae were used in the experiments.

B. bassiana culture

Four isolates of *B. bassiana* were cultured (Fashand, Spt-22, Ir-K-40 and 566) at 25±1°C on potato dextrose agar. After 12 days, conidia were washed with distilled water and concentrations of 10^5 spore/ml were prepared for further testing.

I. farinosae

In winter, hibernated pupae of *H. cunea* were collected from the forests of the Guilan province. Pupae were washed with ethanol 96% and sodium hypochlorite 3% for 2 minutes, respectively. Then they were placed in Petri dishes. The fungus was observed on cultures 1–2 days later. This fungus was identified as *I. farinosae* (code number: 1872c in the fungi collection of the Iranian Research Institute of Plant Protection), identification key of conidiophores was Samson (1974).

Hemocyte characterization by light and phase-contrast microscopy

The hemolymph from ten 4th instar larvae of *H. cunea* was collected for characterization by light microscopy. Larvae were placed in 50°C distilled water for 5 minutes. They were then placed on filter paper. After drying, the larval hemolymph was bled directly onto a clean slide by amputating the foreleg with a pair of micro-scissors. A smear was prepared on each slide with the edge of another glass slide, which was air-dried. Smears were stained with 4% Giemsa stain for 20 min and washed with distilled water. Slides were then washed in water and rinsed in saturated lithium carbonate for 1 minute and finally washed with distilled water, and dried. A permanent slide was prepared in Canada balsam. Ten slides (10 replications) were prepared in this way from 10 untreated individuals, for identifying different haemocyte types based on the identification keys set by Gupta (1979).

Injection of insects with spores

In order to immobilize larvae and to ease the injection, the larvae were chilled on a piece of ice for 10 min, then the body surfaces were sterilized with 70% ethanol. After this, the larvae were injected with 2 µl of 1x10^5 concentration of *B. bassiana*, *I. farinosae* isolates, and latex beads. The carboxylate-modified polystyrene latex beads, 0.3 mm in diameter (aqueous solution, 10% solid content, Sigma Co., St. Louis, MO, USA), were diluted 1:10 in sterile phosphate buffered saline (PBS, pH 7.2) and injected (Borges *et al*. 2008). After injection, larvae were transferred to a rearing box containing mulberry leaves, to follow the course of the assay for further observation. Insects for the control were injected with distilled water.

The effect of fungal spores of *B. bassiana*, and *I. farinosae*, and latex beads on hemocyte number

To determine if injection of spores of *B. bassiana* (4 isolates), *I. farinosae,* and latex beads caused any changes in the levels of total hemocyte numbers and plasmotocyte and granulocyte numbers, 4th instar larvae were injected with 2 µl treatments of a 10^5 spores/ml concentration between the second and third prolegs, similarly the controls were injected with 2 µl of distilled water. Hemolymph was collected 1, 3, 6, 12, and 24 h after injection. Samples of hemolymph from each larva were bled into equal amounts of anticoagulant buffer. Then, the total hemocyte, granulocyte, and plasmotocyte numbers were counted using an improved Neubauer hemocytometer for each treatment.

The effect of fungal spores of *B. bassiana*, and *I. farinosae*, and latex beads on nodulation

Injections were done as mentioned above and nodulation was investigated at 1, 3, 6, 12, and 24 h intervals. Hemolymph was collected from each larva, then samples in 5 replicates were poured into a hemocytometer, and nodules were counted (Franssens *et al*. 2006).

Phenoloxidase activity

To evaluate the effect of *B. bassiana*, *I. farinosae*, and latex beads on Phenoloxidase (PO) activity in the lar-

vae of *H. cunea*, a hemocyte lysate supernatant was prepared after injections were made. Larval hemolymph was mixed with anticoagulant buffer and centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and the remaining pellet was gently washed twice with a phosphate buffer (pH 7 20 mm) (Leonard *et al*. 1985). Cells were homogenized in a phosphate buffer (1 molar) centrifuged at 12,000 rpm for 15 min and the hemocyte lysate supernatant was used in PO assays. Samples were pre-incubated with a buffer at 30°C for 30 min before the addition of L-DOPA (as a substrate). Phenoloxidase activity was measured at 490 nm for 35 minutes. Assays were carried out in 5 replicates.

RESULTS

Five hemocyte types from the hemolymph of *H. cunea* were observed; plasmotocytes, granulocytes, prohemocytes, oenocytoides, and spherulocytes, by phase-contrast microscopy (Fig. 1–3). Prohemocytes are the smallest cells with a round shape and a high nucleus ratio. The nucleus fills the cell, so cytoplasm is placed in a narrow area around the nucleus (Fig. 2). Plasmotocytes are the most numerous of the hemocytes with a polymorphic profile. The most common forms of plasmotocytes are spindle-like with the nucleus located in a central or pericentral position, and relatively homogeneous cytoplasm covered with few granules. Granulocytes have an oval or round shape with a central nucleus, and vary in size. Several granules are located in the cytoplasm (Fig. 1) Oenositoides are small, round or oval cells with a round and obvious nucleus placed by the side of the cytoplasm near the cell membrane. Spherulocytes are small spherical cells, in which the cytoplasm is characterized by the presence of spherical vacuoles (Fig. 3). These cells comprise the lowest numbers in hemolymph of *H. cunea*.
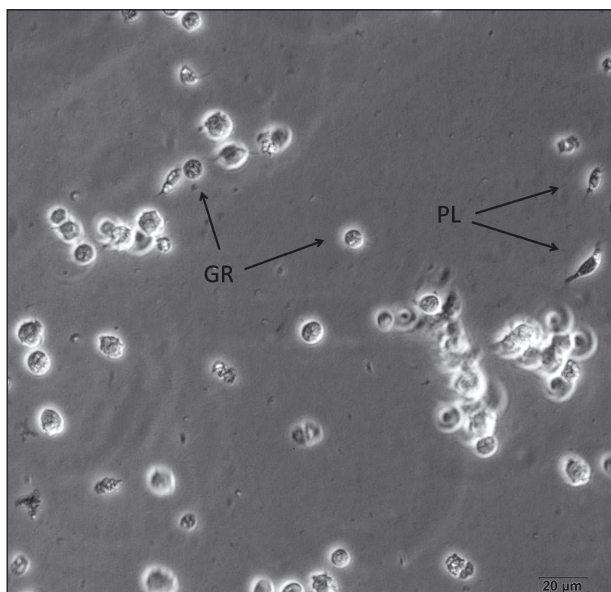

Fig. 1. Phase-contrast microscopy of *H. cunea* hemocytes: arrows indicating plasmotocyte (PL), granulocyte (GR), (20 μm)
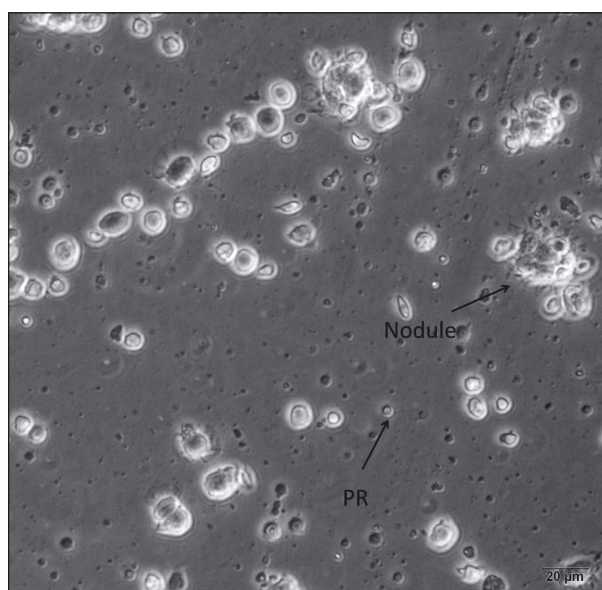

Fig. 2. Phase-contrast microscopy of nodule formation (challenge between hemocytes and spores) and also showing Prohemocytes (PR) in *H. cunea* (20 μm)
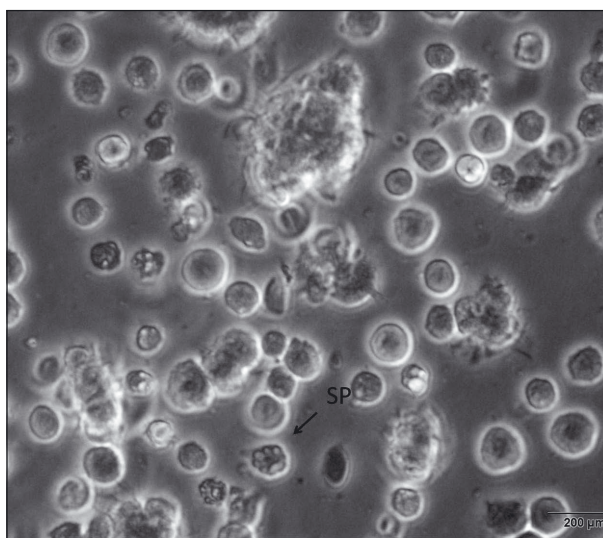

Fig. 3. Phase-contrast microscopy of hemocytes in *H. cunea* arrows indicating Spherolucytes (SP) (20 μm)

Effect of *B. bassiana*, *I. farinosae* spores, and latex bead on total hemocyte, plasmotocyte, granulocyte, and oenocytoide counts of *H. cunea* hemocyte

The total hemocyte of *H. cunea* larvae showed significant differences in various intervals after injection of *B. bassiana*, *I. farinosae,* and latex beads, in comparison with the controls. However, profiles of plasmotocytes, granulocytes, and oenocytoides significantly changed after immune tests, in all treatments, compared with the control.

Figure 4 shows that the highest number of total hemocytes varied according to the time duration after treatments. Fashand had the highest total, plasmotocyte, granulocyte, and oenocytoide numbers 6 h after injection (Fig. 4–7). But the Spt-22 isolate had the highest change of total hemocyte, granulocyte, plasmotocyte, and oenocytoide counts at 12 h post injection. Ir-K-40 isolate and 1872c had the same effect on the process of cell modification.

Both Ir-k and 1872c increased hemocyte, plasmotocyte, granulocyte, and oenocytiode totals 3 h after injection. Then, all of these above parameters decreased gradually, so that the lowest number was observed 24 h after injection. Immune reaction of *H. cunea* against isolate 566, and latex beads was different from that of other fungi. Total

hemocyte, granulocyte, plasmotocyte, and oenocytoide numbers 24 h after the injection of latex beads increased significantly (Fig. 4–7). In those larvae that had been treated with isolate 566 (isolated from *Eurygaster integriceps* in Esfahan province), and latex beads, immune activity was postponed in comparison to other treatments (Fig. 4–7).
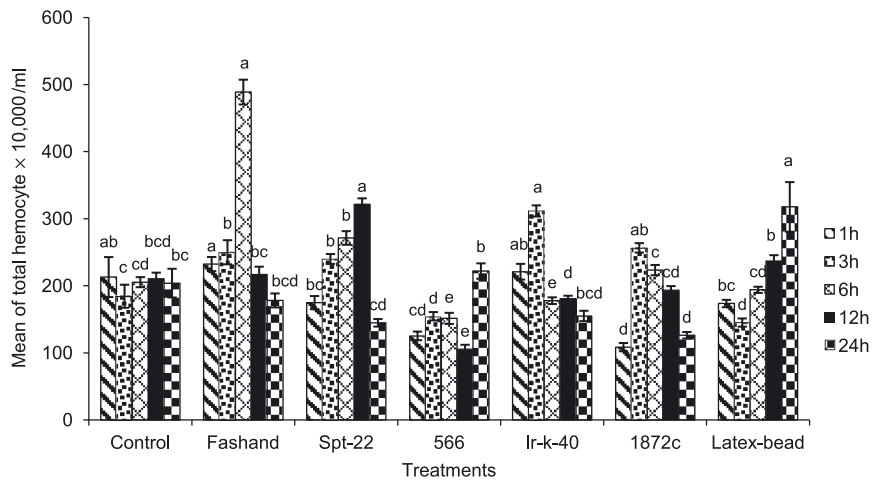

Fig. 4. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex beads on the total hemocyte x 10⁴/ml /a, b, c and d means significant at p ≤ 0.05
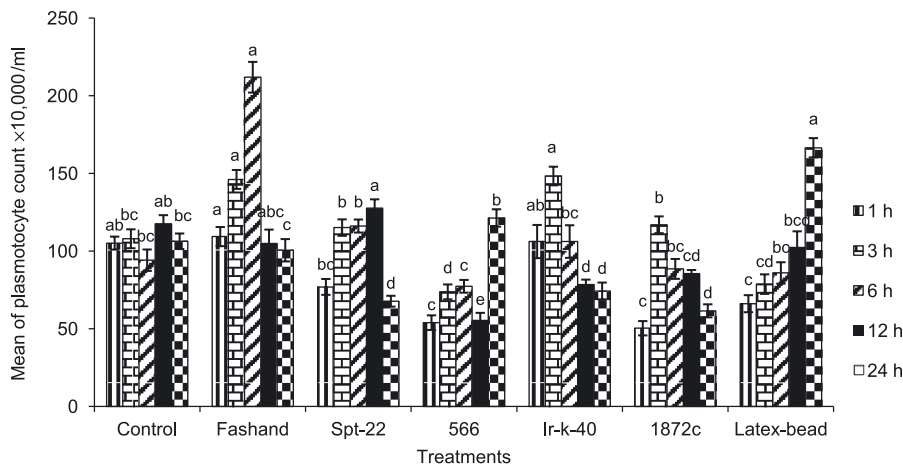

Fig. 5. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex beads on the plasmotocyte count x 10⁴/ml/ /a, b, c and d means significant at p ≤ 0.05

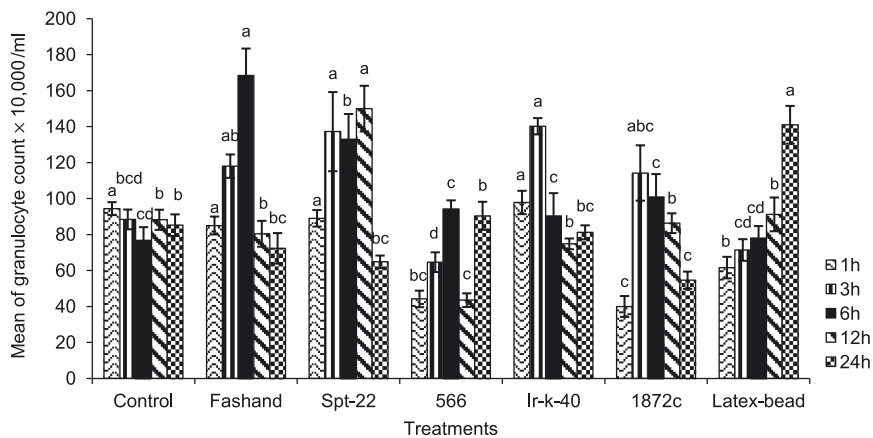Fig. 6. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex beads on the granulocyte count x 10⁴/ml /a, b, c and d means significant at p ≤ 0.05
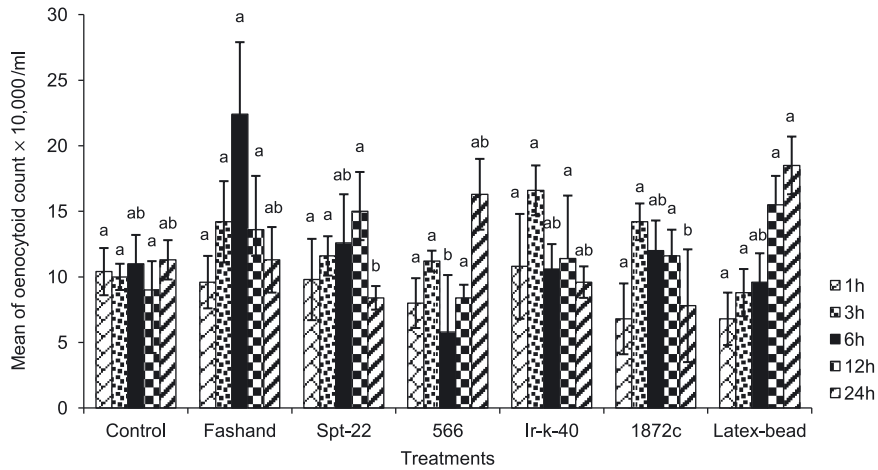
Fig. 7. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae*, and Latex beads on the oenocytoid count x 10⁴/ml/
       a, b, c and d means significant at p ≤ 0.05

Effect of *B. bassiana*, *I. farinosae* spores, and latex beads on phagocytosis

Results showed that plasmotocytes and granulocytes of *H. cunea* played an important role in phagocytosis against foreign particles. The most phagocytosis occurred at 30 and 60 min after introducing spores to hemolymph, in all treatments (Fig. 9, 10). After 30 min this test was higher than after 60 min. Both plasmatocytes and granulocytes presented phagocytic activity 90 min after an injection of latex beads. It seems that after 90 min, engulfment of fungal spores by hemocytes sharply decreased (Fig. 9, 10). Prohemocyte count decreased due to phagocytosis. It means that they probably modified to immunocytes (Fig. 9, 10).
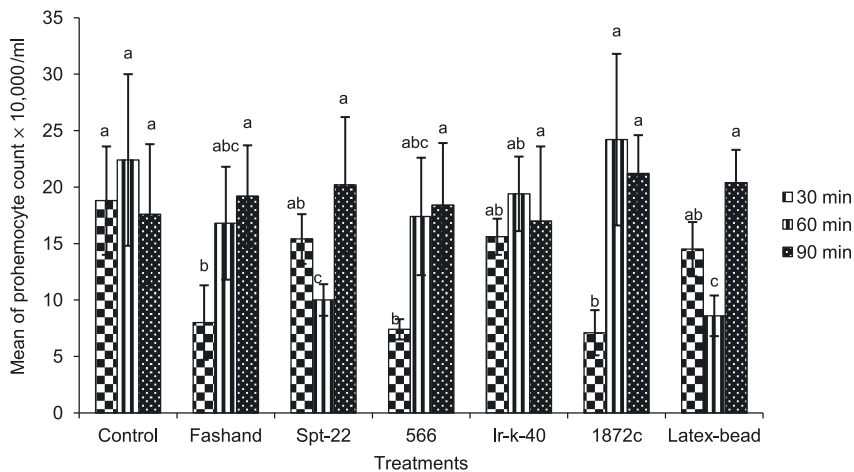

Fig. 8. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae*, and Latex beads on the prohemocyte count in phagocytosis x 10⁴/ml
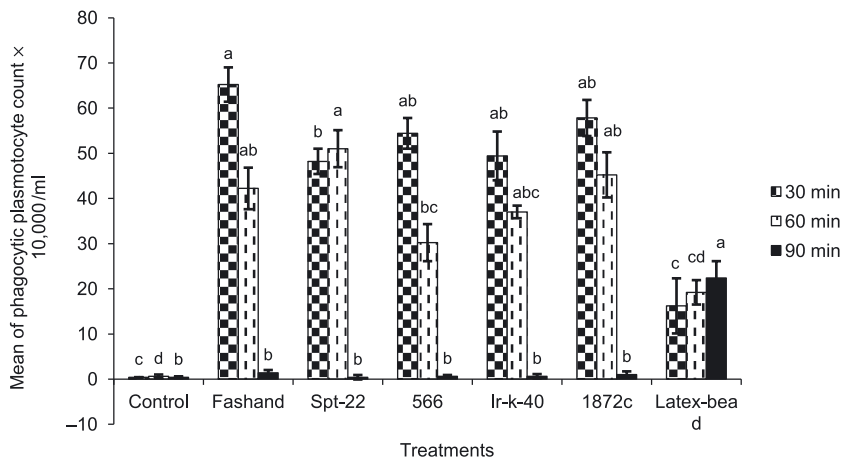   /a, b, c and d means significant at p ≤ 0.05

Fig. 9. Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosa,e* and Latex beads on the phagocytic plasmotocyte x 10⁴/ml
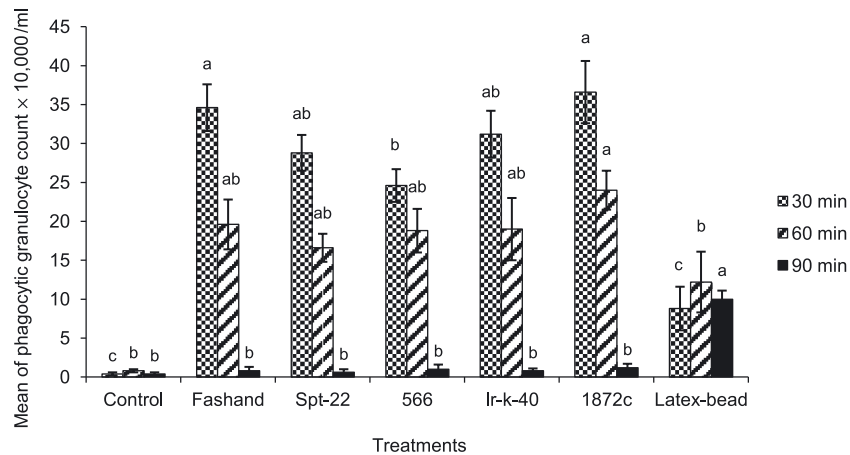   /a, b, c and d means significant at p ≤ 0.05

Fig. 10.   Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex bead on the phagocytic granulocyte x 10^4/ml/ a, b, c and d means significant at p ≤ 0.05

Effect of *B. bassiana*, *I. farinosae* spores, and latex beads on nodulation

Nodulation has been observed after phagocytosis in immune reactions of *H. cunea* against *B. bassiana*, *I. farinosae*, and latex beads (Fig. 11). In all treatments, the highest number of nodules was observed three and six hours after injection. After 12 and 24 h, nodule formation sharply decreased in all treatments including the injection with latex beads treatment (Fig. 11). So, most cellular defense of *H. cunea* occurred in the first hours of inoculation by fungi.

Effect of *B. bassiana*, *I. farinosae* spores, and latex beads on PO activity

Injection of fungi and latex beads in *H. cunea* larvae activated the PO system, during intervals after inoculation (Fig. 12). The Fashand isolate demonstrated the highest PO activity after 3 and 6 h intervals. The highest activity of PO was 12 h after the injection of the Spt isolate; this parameter was highest 3 h after injection of Ir-k and *I. farinose*. The highest activity of PO was observed 24 h after the injection of both isolate 566, and latex beads (Fig. 12). A direct correlation was observed between total hemocyte and PO activity in defense reactions of *H. cunea* against 4 isolates of *B. bassiana*, *I. farinosae,* and latex beads.
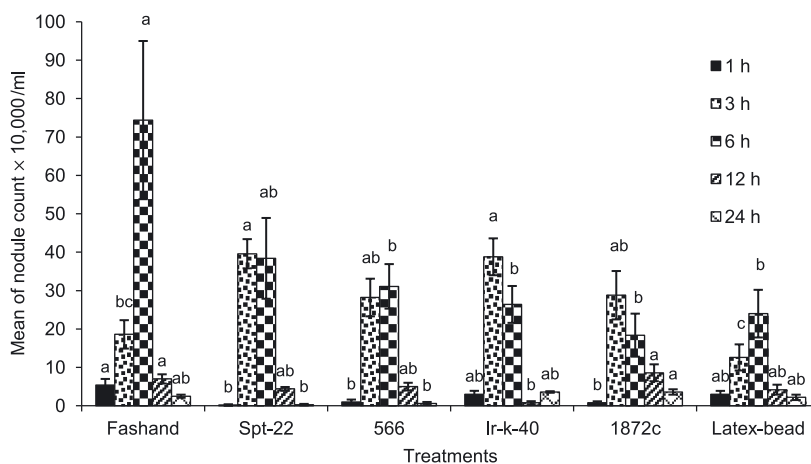

Fig. 11.   Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex beads on nodule formation x 10^4/ml/ a, b, c and d means significant at p ≤ 0.05
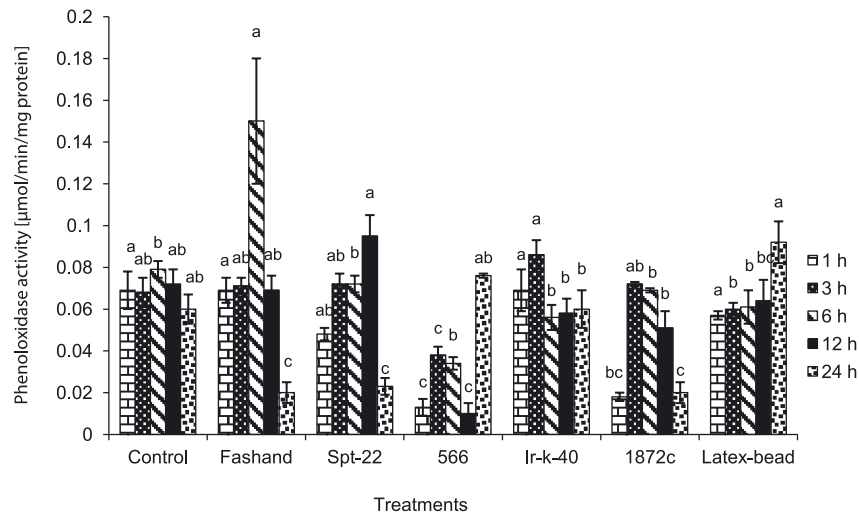
Fig. 12.   Effect of 4 isolates of *B. bassiana*, 1 isolate of *I. farinosae,* and Latex beads on phenoloxidase activity/
a, b, c and d means significant at p ≤ 0.05

DISCUSSION

H. cunea is one of the most significant pests causing defoliation in Guilan province of Iran as well as in other areas (Yarmand *et al.* 2009). An evaluation of the interaction of pathogens *e.g. B. bassiana* and cellular immune reactions of this pest, can provide new information and methods applicable for control programs. The total number of circulating hemocytes in an insect vary according to the developmental and physiological stage (Sanjayan *et al.* 1996; Manachini *et al.* 2011). In this study, hemocyte types of 4th instar larvae of *H. cunea,* total hemocyte, granulocyte, and plasmotocyte numbers were studied after using 4 isolates of *B. bassiana*; one isolate of *I. farinosae,* and latex beads. Both *B. bassiana* and *I. farinosae* are general entomopathogenic fungi. Currently their application in integrated pest management is commonplace.

In this research, there was a significant increase in the total hemocytes, plasmotocyte, and granulocyte numbers observed after injection of the isolates of *B. bassiana*, and *I. farinosae,* but then the number declined. In Latex-treated insects, all the above factors increased 24 h after injection. These results were similar to those of Moushumi *et al.* (2008) concerning the interaction of *Dicladispa armigera* (Olivier) hemocytes with *B. bassiana.* The total granulocyte and plasmotocyte numbers also increased 12 h after treatment by *Metarhizium anisopliae* (Metchnikoff) in *Oxya japonica* (Anggraeni *et al.* 2011). Nappi (1981) reported an increase in hemocyte number in response to a braconid *Asobara tabida* (Nees) (Hymenoptera: Braconidae) and attributed this to an activated defense response in the insect against an invading parasitoid. Hoch *et al.* (2004) reported a significant increase in total hemocyte count (THC) of *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) infected by an entomopathogenic microsporidia, *Vairimorpha* sp. (Microsporidia: Burenellidae). The total hemocyte count and differential hemocyte count increased 3 h after the injection of *B. bassiana* into the hemolymph of *E. integriceps* (Zibaee *et al.* 2011). However, interaction between the pathogen-immune reaction may lead to a decline of hemocytes. It is reported that total hemocyte levels in

Melanoplus sanguinipes (Fabricius), *Schistocerca gregaria* (Forskal) and *Rhynchophorus ferrugineus* decreased during fungal and bacterial infection (Bidochka and Khachatourians 1987; Gunnarsson 1988; Manachini *et al.* 2011).

Previous studies by other researchers found a major influence of pathogenesis on DHC in *Dysdercus koenigii* (Fab.) (Heteroptera: Pyrrhocoridae) (Tikku *et al.* 1992) and *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) (Mochiah *et al.* 2003). These studies reported a significant increase in numbers of granulocytes and oenocytoides in the hemolymph of infected insects but a decrease in prohemocytes and plasmatocytes.

In this study, a significant increase in the number of plasmotocytes, granulocytes, and oenocytoides in larvae injected with fungal spores, and latex beads in intervals after inoculation, showed that these cells have a key role in the immune system of this insect against foreign particles.

Immune reactions of *H. cunea* against *B. bassiana,* and against *I. farinosae* showed that plasmotocytes and granulocytes are the main factors in phagocytosis and nodulation. In this study, the most phagocytosis activity occurred 30 and 60 min after injection, in all treatments. It appears that later, granulocytes and plasmotocytes indulge in nodule formation and PO activity. Phagocytosis refers to the engulfment of entities by an individual cell. Hemocytes can induce phagocytosis in both biotic targets such as bacteria, yeast, and pathogens. Hemocytes can also induce phagocytosis in abiotic targets such as synthetic beads (Yokoo *et al.* 1995; Hernandez *et al.* 1999; Da Silva *et al.* 2000). The hemocyte types responsible for phagocytosis are different among invertebrates and even among insect taxa. In Lepidoptera, granulocytes and plasmatocytes are the only hemocyte types reported to be phagocytic (Jiravanichpaisal 2006). Prohemocytes are stem cells that form plasmotocytes and granulocytes, so their numbers decreased due to immune activity (Yamashita and Iwabuchi 2001; Ling *et al.* 2005).

Nodulation occurred in the first hours of treatments. Similar results were observed by Zibaee *et al.* (2011) in

E. integriceps. Plasmotocytes and granulocytes assembled around spores and latex particles, then the spores and latex particles were removed by the melanization process. Both phagocytosis and nodulation were key factors in the immune activity in *H. cunea*.

Phenoloxidase (PO) is a copper-containing enzyme that catalyzes the oxygenation of monophenols to *o*-diphenols and *o*-diphenols to *o*-quinones (Mason 1965). These reactions are key steps in the synthesis of the black pigment melanin, which is often seen on host cuticles after infection surrounding the capsules and nodules of parasitized hosts. Phenoloxidase is present in the hemolymph as an inactive zymogen, prophenoloxidase (PPO). The activation of PPO is a response to stress as a result of microbial invasion (Ashida and Brey 1998). Since hemocytes are one of the production sources of prophenoloxidases, and in lepidopterans, oenocytoids are identified as the cell-type producing PPO (Iwama and Ashida 1986; Jiang *et al*. 1997), so total hemocyte increase may lead to increasing PO activity after the injection of isolates of *B. bassiana*, *I. farinosae,* and latex beads. Several studies have shown that PO levels are elevated in response to injection of fungal components such as blastospores and conidiospores (Gillespie and Khachatourians 1992; Hung and Boucias 1996). Gillespie *et al*. (2000) have shown that a topical application of conidia from *M. anisopliae* var. *acridum* results in elevated PPO in the hemolymph. This demonstrates that the host can respond to infection by increasing levels of PPO available for defense processes. It has also been reported, that infection of *Spodoptera exigua* (Hubner) with *B. bassiana* caused marked alterations in the levels and distribution of PO activity (Hung and Boucias 1996). Conversely, Bidochka and Hajek (1998) observed PPO activation in *Lymantria dispar* (Linnaeus, 1758) challenged with pathogens.

In the present study, the interaction between immune responses and pathogenic agents suggests that the cellular defense of *H. cunea* is competitive enough to overcome pathogenic activity within 24 hours. Further research is needed for the evaluation of this competence, in more intervals after injection of foreign particles.

ACKNOWLEDGEMENTS

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