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RESEARCH REPORT

Quantitative comparison for some immune responses among *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio* against the entomopathogenic fungus *Beuveria bassiana*

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Abstract

Some defense reactions including cuticular lipids, phagocytic activity, nodulation and hemolymph phenoloxidasec activity were compared in *Eurygaster integriceps*, *Ephestia kuehniella* and *Zophobas morio* exposed to five different *B. bassiana* isolates. The cuticular lipids were stimulating on conidia germination possibly with no fungicidal or fungistatic ability in epicuticular fatty acids. There was correlation between all studied immune system reactions and the virulence of isolates to *E. kuehniella* and *E. integriceps*. DE as a less virulent fungal isolate stimulated immune reactions in high levels in most experiments, despite of TV as the most virulent isolate. *E. integriceps* was the most sensitive host with lowered immune reactions. *Z. morio* showed resistant with the highest nodulation. *E. kuehniella* had moderate sensitivity with maximal phagocytic and the phenoloxidase activity. The phagocytic activity was the highest at 30 min after fungus injection. The nodulation and the phenoloxidase activity were demonstrated at 6, 12 and 24 h after injection. The most nodulation rate was observed at 24 h. The phenoloxidase activity for the most isolates reached a maximum value for *Z. morio* and *E. integriceps* after 12 h and in *E. kuehniella* after 24 h. Our research provides an interesting perspective in host susceptibility to fungal infections.

Key Words: *B. bassiana; E. integriceps; E. kuehniella; Z. morio*; immune system

Introduction

There are different defense barriers in insect body which entomopathogenic fungi should have an ability to overcome them. They infect insects by penetrating the cuticle, thus insect cuticle plays a very important role as a barrier to infection in host specificity (Murrin and Nolan, 1987; Altre and Vandenberg, 2001). Not only chemical and physical toughness, but also the cuticular compounds have been demonstrated to have toxicity to invading fungi (Ortiz-Urquiza and Keyhani, 2013). Furthermore, insects have a well-developed innate immune system that allows a general and rapid response to infectious agents. The innate immune system of insects relies on both humoral and cellular responses. Humoral immune responses include several antimicrobial peptides, enzymic cascades that regulate coagulation and melanization of haemolymph, and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The term cellular immune response refers to hemocyte-mediated responses, including phagocytosis, nodulation and encapsulation (Lavine and Strand, 2002). Phenoloxidase is an oxidoreductase that catalyses the oxidation process of phenols present in the hemolymph to cytotoxic quinones (Ashida, 1990). These quinones polymerize non-enzymatically to melanin. Both quinones and melanin are toxic to microorganisms (Soderhall and Cerenius, 1998). The cellular reaction is started with phagocytosis. During the phagocytic process, cells ingest and eventually destroy foreign particles (Rosales, 2011). Nodule formation is initiated with the micro-aggregation of hemocytes, which entrap large numbers of microorganisms. These micro-aggregates grow in size by recruiting additional hemocytes. Finally, the process ends with melanization into darkened nodules, which attach to the body wall or to various internal organs (Franssens *et al.*, 2006).

Variations in the susceptibility of insect species to fungal invasion may result from their immune potencies. Reaction of insect immune system to fungal infection has been studied earlier, for example

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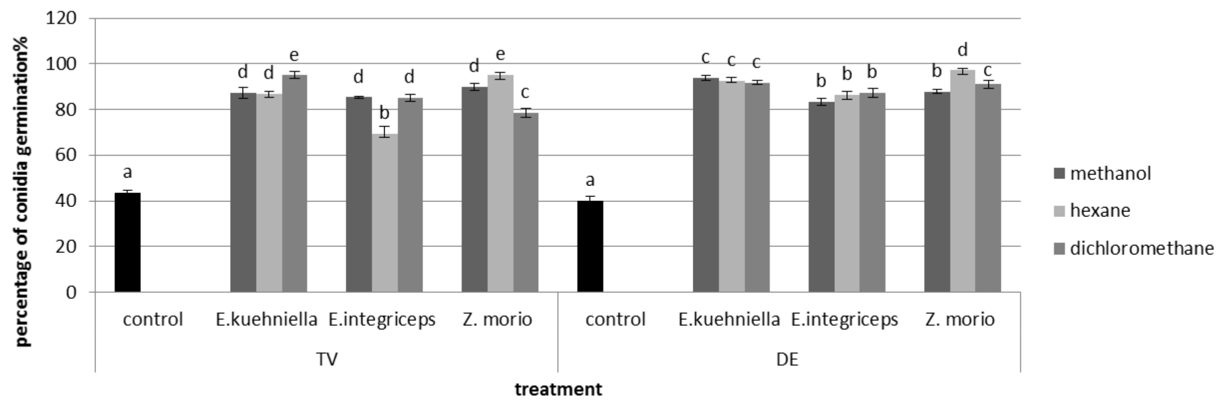


Fig. 1 Effect of different cuticular extract on mean (±SE) percentages of TV and DE isolates spore germination. Means in columns marked with different letters are significantly different (F-LSD, $p < 0.05$)

Lee *et al.* (2005) investigated cellular immune responses in the larvae of *Mamestra brassicae* injected with three entomopathogenic fungi, *Beauveria bassiana*, *Nomuraea rileyi* and *Paecilomyces tenuipes*, suggesting that the different virulence of each fungal species is caused by specific immune responses in the larvae. Zibaee *et al.* (2014) demonstrated different levels of cellular immune response of *Chilo suppressalis* against several entomopathogenic fungi including isolates of *B. bassiana*, *Metarhizium anisopliae*, *Isaria fumosoroseus* and *Lecanicilium lecanii*. Most researches have considered lepidopteran hosts (Meshrif *et al.,* 2011; Ajamhassani *et al.,* 2013; Khosravi *et al.,* 2014), but comparative studies among different insect species are not many. Bogus *et al.* (2010) studied immune responses of three insect species including *Dendrolimus pini, Galleria mellonella* and *Calliphora vicina* to the entomopathogenic fungus *Conidiobolus coronatus*. Our data describes and compares some different defense criteria including cuticular lipids, phagocytic activity, nodulation and hemolymph phenoloxidase activity in three host species from different insect orders. Sunn pest, *Eurygaster integriceps*, the flour moth, *Ephestia kuehniella* and giant mealworm or superworm *Zophobas morio* were compared for their immune reactions against five different *B. bassiana* isolates.

Materials and Methods

Insects rearing

Sunn pest adults were collected from wheat fields of Varamin, Tehran province, Iran. They reared in plastic boxes (30×30×50 cm) on wet wheat seeds (*Triticum aestivum* var. Pishtaz), and a piece of cotton soaked with water was used as a water source. Folded strips of paper were hung in containers for oviposition. After hatching of eggs, nymphs were transferred to plastic shelves with pots of wheat and wet wheat seeds. Fifth instar nymphs were used in experiments. The flour moth was prepared from colony in Biological Control Laboratory in College of Agriculture and Natural Resources, University of Tehran and bred in plastic containers containing flour and yeast (10 g yeast per kg of flour), the fourth instar larvae were used in experiments. Initial colony of *Z. morio* was obtained from a pet store and reared in plastic containers containing wheat bran and pieces of potato. The last instar larvae of new generation were used in experiments. The rearing condition was 25 ± 1 ºC, 70 ± 5 % R.H. and 16:8 (L:D) h photoperiod for all insects.

Beauveria bassiana *isolates*

Five *B. bassiana* isolates encoded TV, OZ, UN, DV and DE (soil origin) were grown on Sabouraud Dexterose Agar (SDA) and maintained at 25 ± 1 °C, 70 ± 5 % RH, and a photoperiod of 16:8 (L:D) h. Cultures were scrapped after sporulation and conidia were obtained (Goettel and Inglis, 1997). Virulence of these isolates have been studied previously (our unpublished data) on these insects, as TV and DE were most and less virulent isolates while the other has moderate virulence on *E. kuehniella* larvae and *E. integriceps* nymphs (22.70 ± 1.39 and 32.5 ± 1.71 % mortality for DE, 36.87 ± 2.82 and 64.17 ± 1.54 % for DV, 45.74 ± 1.84 and 66.67 ± 1.61 % for UN, 58.43 ± 1.90 and 71.67 ± 1.67 % for OZ, 89.15 ± 2.39 and 86.00 ± 1.53 % for TV on *E. kuehniella* and *E. integriceps*, respectively). None of the isolates affected the survival of *Z. morio* larvae.

Labeling of B. bassiana *conidia*

B. bassiana conidia were obtained from the 10 - 14 days old culture on PDA. These conidia were 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). They were then washed and re-suspended in a sterile CO3-HCO3 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 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).

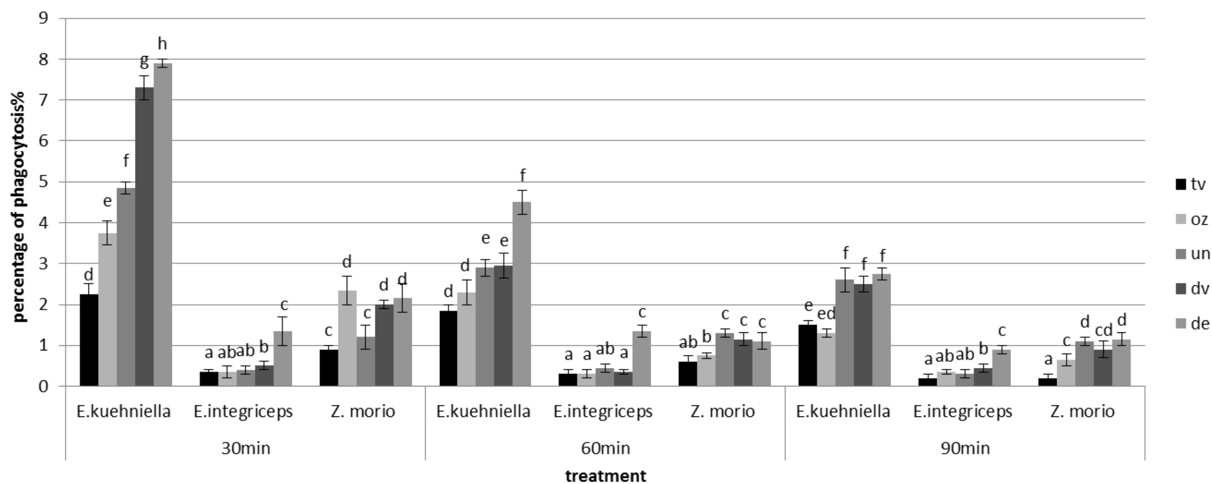


Fig. 2 Effect of *B. bassiana* isolates conidia on mean phagocytosis percentage (±SE) in different insect on different times, means followed by different letters differ significantly (F-LSD, $p < 0.05$)

Extraction of cuticular surface lipids

Insects were killed by freezing and washed several times with distilled water. After air drying, they were immersed in three different solvents separately, methanol (polar solvent), hexane (non-polar solvent) and dichloromethane (solvent with intermediate polarity) for 30 min. The insects were picked up and the extracts were collected in glass Petri dishes, dried in a hood and resolved in ethanol 96 %. After alcohol evaporation, residuals was weighted and solved in ethanol 96 % again. The extracts were maintained in freezer at -20 °C (Wang and St. Leger, 2005).

Effect of cuticular extract on conidia germination

The Petri dishes (9 cm diameter) containing thin layers of water agar (0.5 %) were inoculated with 150 µl of fungus suspension 10^6 (conidia/ml) of isolates TV and DE. One hundred microliters of each extract (4 mg/ml) were pipetted on glass coverslips. After solvent evaporation, they were placed up-side down on the surface of the media in the Petri dishes. Glass coverslips without extracts were used as control. Percentages of germinated conidia were estimated after 24 h under a light microscope at 40x magnification. Three replicates were considered for each treatment.

Phagocytosis assay

Insects were immobilized in freezer for some min and were surface sterilized with ethanol 70 %. Quantities of 20, 1 and 1 µL of 1×10^8 conidia/ml of fungal concentration (labeled conidia), were injected to giant mealworm larvae (between second and third thoracic segment), Sunn pest nymph (between second and third legs) and flour moth (lateral abdominal section), respectively by a Hamilton syringe. The treated insects were transferred to rearing containers. Control larvae were injected with distilled water. Insect hemolymph was collected at different intervals (15, 30, and 60 min post injection). Insects were anesthetized on ice, surface sterilized with ethanol (70 %) and bled by cutting one of the prolegs for Sunn pest nymphs and giant mealworm larvae or scratched body surface for flour moth larvae. The hemolymph was then collected and mixed with ice-cold anticoagulant solution buffer (0.098 M NaOH, 0.186 M NaCl, 0.017 M EDTA, 0.041 M citric acid, pH 4.5) in 30:70 ratio (Anggraeni and Ratcliffe, 1991). Phagocytic activity was determined by counting the cells containing conidia in a Neubauer hemocytometer Observations were done on a Zeiss florescent microscope. Hemolymph samples were checked in 3 replicates and whole experiment was replicated one more time.

Effect of fungal conidia on nodulation

Quantities of 10, 0.5 and 0.5 µL of 1×10^6 conidia/ml of fungal concentration (labeled conidia) were injected according to the above mentioned method. Hemolymph was collected at 6, 12, and 24 h after injection from chilled, surface sterilized (ethanol 70 %) larvae. Ten microliter samples of hemolymph from each insect were mixed with 10 µL cold anticoagulant buffer. Then, the number of nodules was counted under a light microscope at 20x magnification. Hemolymph samples were checked in 3 replicates and whole experiment was replicated again.

Assay for PO activity

Injection was the same as nodulation assay. According to Hung and Boucias (1996), 20 µL of collected haemolymph was mixed with the same volume of phosphate buffer (pH 7) at 4 °C. The samples were centrifuged (12,000×*g*) at 4 °C for 5 min. The supernatant (plasma) was collected and used for PO activity. The phenoloxidase assays were carried out in 96-well plates. Each well contained 20 µl enzyme, 20 µl substrate (20 mM L-DOPA) and 80 µl phosphate buffer, pH 7.0. The absorbance was recorded by a micro plate reader (BioTek, USA) over 30 min with a one min interval at 490 nm. This assay was carried out with three replications for each treatment and the whole assay was repeated.

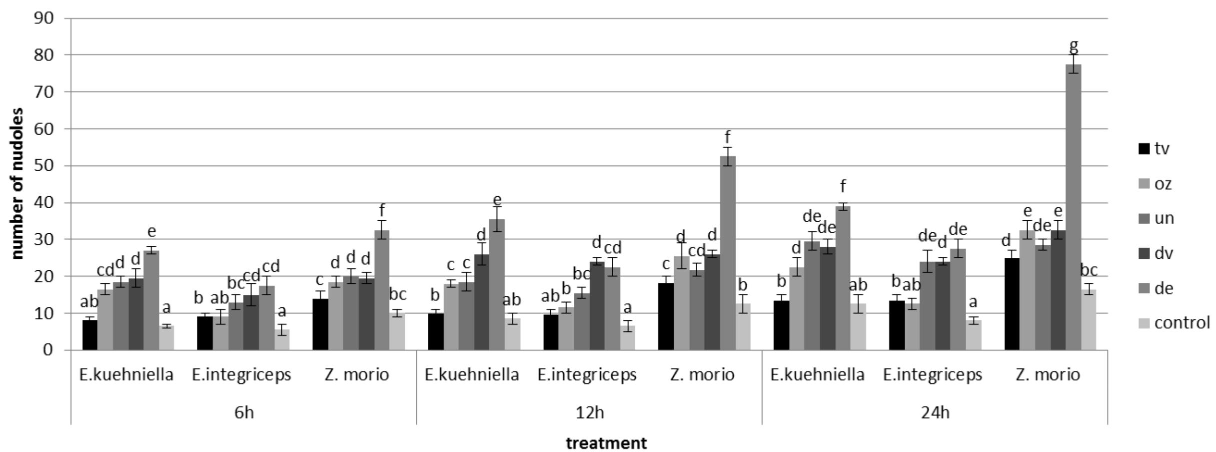


Fig. 3 Effect of *B. bassiana* isolates spores on mean number of nodule formation (±SE) in different insect on different times, means followed by different letters differ significantly (F-LSD, $p < 0.05$)

Protein determination

The protein content of the samples was determined according to Lowry *et al.* (1951) using bovine serum albumin (Bio- Rad) as the standard.

Statistical analysis

All experimental data were subjected to analysis of variance (ANOVA) ($p < 0.05$). Pooled data of two-time repeats of the whole assay were analyzed to determine possible significant differences among the treatments via F-LSD test post-significant ANOVA. Possible correlation between immune reactions and virulence of isolates was analyzed through Pearson correlation coefficient (SAS Institute, 2002).

Results

Effect of cuticular extract on conidia germination

According to results (Fig. 1) all extracts (4 mg/ml) had stimulating effect on conidia germination and hexane extracts had significantly less effect compared with two others ($F_{3, 36} = 759.05$, $p < 0.0001$). There was no statistical difference between two isolates ($F_{1, 36} = 0.00$, $p = 0.97$), but the cuticular extracts of *Z. morio* had the highest stimulating effect on the conidia germination, while cuticular extracts of Sunn pest had less effect ($F_{3, 36} = 50.58$, $p < 0.0001$). However, there was no correlation between conidia germination in cuticular extracts and virulence of isolates ($p > 0.05$).

Phagocytosis assay

With increasing time, percentage of phagocytosis was decreased and the most phagocytic activity was observed 30 min after injection (Fig. 2). There were significant differences among the isolates at 30 min ($F_{4, 15} = 48.59$, $p < 0.0001$), 60 min ($F_{4, 15} = 27.80$, $p < 0.0001$) and 90 min ($F_{4, 15} = 3.02$, $p < 0.0001$) and between insects at 30 min ($F_{2, 15} = 436.89$, $p < 0.0001$), 60 min ($F_{2, 15} = 251.36$, $p < 0.0001$) and 90 min ($F_{2, 15} = 23.99$, $p < 0.0001$). As, the highest and lowest percentages were for *E. kuehniella* larvae and *E. integriceps* nymphs, respectively. Isolate DE had the highest effect. There was a negative correlation between the percentage of phagocytosis and the virulence of isolates on *E. kuehniella* larvae at 30 min (r= 0.91, $p = 0.0002$), 60 min (r = 0.84, $p = 0.0019$) and 90 min (r = 0.69, $p = 0.026$) and on *E. integriceps* at 30 min (r = 0.82, $p = 0.0030$), 60 min (r = 0.90, $p = 0.0003$) and 90 min (r = 0.91, $p = 0.0002$).

Effect of fungal conidia on nodulation

With increasing time, the number of nodules was increased and the most nodulation was observed 24 h after injection (Fig. 3). There were significant differences between the isolates at 6 h ($F_{5, 18} = 35.55$, $p < 0.0001$) , 12 h ($F_{5, 18} = 70.01$, $p < 0.0001$) and 24 h ($F_{5, 18} = 98.37$, $p < 0.0001$) and between insects at 6 h ($F_{2, 18} = 25.04$, $p < 0.0001$), 12 h ($F_{2, 18} = 45.13$, $p < 0.0001$) and 24 h ($F_{2, 18} = 97.84$, $p < 0.0001$), the highest and lowest numbers of nodules were for *Z. morio* larvae and the *E. integriceps* nymphs, respectively. Isolate DE had the highest effect. There was a negative correlation between the number of nodules and the virulence of isolates for two insects, *E. kuehniella* larvae at 6 h (r = 0.94, $p < 0.0001$), 12 h (r = 0.92, $p = 0.001$) and 24 h (r = 0.94, p < 0.001) and *E. integriceps* at 6 h (r = 0.73, $p = 0.0144$), 12 h (r = 0.710, $p = 0.0197$) and 24 h (r = 0.72, $p = 0.0177$).

Assay of phenoloxidase (PO) activity

Phenoloxidase activities were statistically different among the isolates at 6 h ($F_{5, 18} = 8.76$, $p = 0.0003$), 12 h ($F_{5, 18} = 3.01$, $p = 0.04$) and 24 h ($F_{5, 18} = 0.04$, $p = 0.6990$). Moreover, the phenoloxidase activities of the insects varied significantly at 6 h ($F_{2, 18} = 8.04$, $p = 0.0035$), 12 h ($F_{2, 18} = 16.72$, $p < 0.0001$) and 24 h ($F_{2, 18} =45.13$, $p < 0.0001$); as the enzyme activity reached a maximum value in 12 h for *Z. morio* and the *E. integriceps* and in 24 h for *E. kuehniella* (Fig. 4). There was correlation between the phenoloxidase (PO) activity and the virulence of isolates for two insects, *E. kuehniella* larvae at 6 h (r

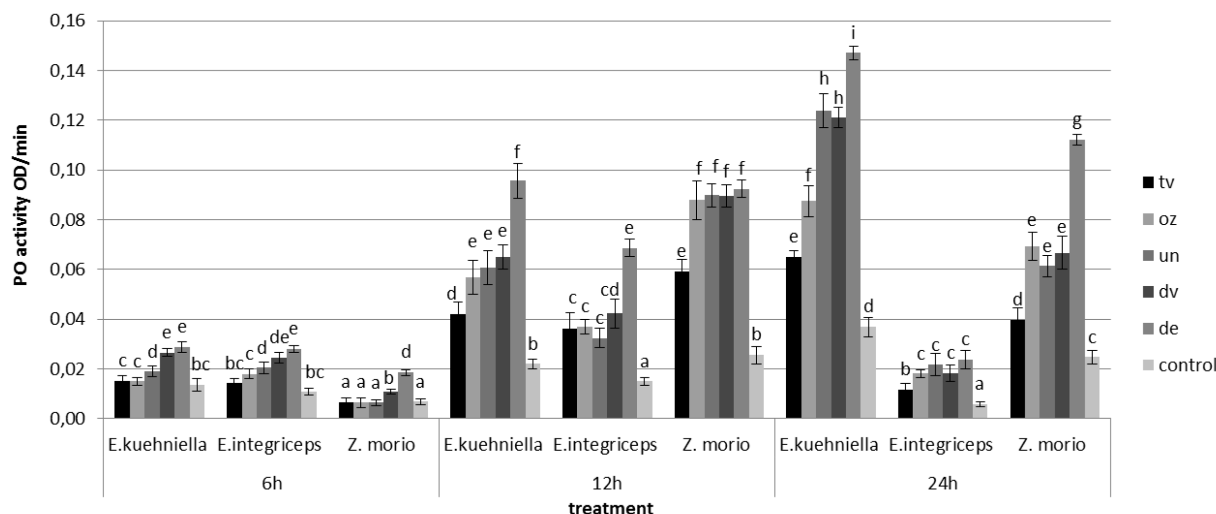


Fig. 4 Effect of *B. bassiana* isolates spores on mean phenoloxidase (PO) activity (±SE) in different insect on different times, means followed by different letters differ significantly (F-LSD, $p < 0.05$)

= 0.80, $p < 0.0051$), 12 h (r = 0.81, $p = 0.0044$) and 24 h (r = 0.92, $p < 0.001$) and *E. integriceps* at 6 h (r = 0.87, $p = 0.0010$), at 12 h (r = 0.65, $p = 0.0404$) and at 24 h (r = 0.71, $p = 0.0209$).

Discussion

To clarify the relationship between host susceptibility and virulence of *B. bassiana* isolates, some defense reactions including cuticular lipids, phagocytic activity, nodulation and hemolymph phenoloxidasec activity in 3 different insect were compared against five different *B. bassiana* isolates. Insects were selected according to our pervious study, Sunn pest *E. integriceps* with high level sensitivity, the flour moth *E. kuehniella* with moderate sensitivity and giant mealworm *Z. morio* as resistancec host to fungal infection. *B. bassiana* isolates were TV (the most virulent), OZ, UN, DV (moderate virulent) and DE (the less virulent).

Insect epicuticle represents the first barrier to entomopathogenic fungi infection, its components are extremely heterogeneous and therefore have the potential to lead to different pathogen responses in particular insects (Thompson, 1973; Wang and St. Leger, 2005). Inhibition effect of epicuticular fatty acids on conidia germination has been reported previously (Smith and Grula, 1982; Saito and Aoki, 1983; Bogus *et al.,* 2010; Urbanek *et al.,* 2012). In current research, three solvents with different polarity were used for lipid extraction and all of them were stimulating on conidia germination. TV and DE isolates were used for this stage of experiment and no difference was shown between them. The fungi isolates were not specific pathogens on three insects from this point of view. While, *Z. morio* was resistant to infection by fungal isolates, as a result, it seems that there is no fungicidal or fungistatic ability in epicuticular fatty acids of these insects. In contrast, Wang and St. Leger (2005) showed that germination and appressorial formation of the specific locust pathogen, *M. anisopliae* var. *acridum* was completely down with locust *Schistocerca gregaria* cuticular extract compared to other insects such as *Leptinotarsa decimlineata* and *Magicicada septendecim* as non-specific hosts. As biochemistry of *E. integriceps*, *E. kuehniella* and *Z. morio* epicuticules are not clear, possibly other components except the fatty acids have inhibitory effects on fungal infection. Of course supplementary studies are needed.

Immune system reactions were significantly different among insects and fungal isolates. Our data showed that the most phagocytic activity (Fig. 5) 30 min after fungus injection in all treatments; the lowest percentage was observed in *E. integriceps*. It seems that fungi could overcome to the immune reaction of sunn pest, because the reaction level is low and without remarkable changes in the time intervals. Also, Ajamhassani *et al.* (2013) reported that phagocytic activity of conidia of *B. bassiana* and *I. fumosorosea* isolates in *Hyphantria cunea* larvae was reduced during the time. But, Khosravi *et al.* (2014) showed increase in phagocytic activity of *B. bassiana* conidia in *Glyphodes pyloalis* larvae during the time.

We studied nodule formation in three times 6, 12 and 24 h after injection of fungal conidia. Aggregation of hemocytes and entrapping of labeled conidia was seen and melanization was occurred during the time (Fig. 6). The formation of nodules was increased during the time and the highest number of nodules was recorded for *Z. morio*. Microscopic examination showed that its nodules melanized extremely in comparison with them in other insects. Therefore, the beetle has better performance than others in nodule formation. Zibaee *et al.* (2011) demonstrated the most number of nodules were observed 3 h after injection of *B. bassiana* conidia in *E. integriceps* adults, while our studies showed the maximum number of nodules after 24 h fungus injection in fifth instar nymphs.

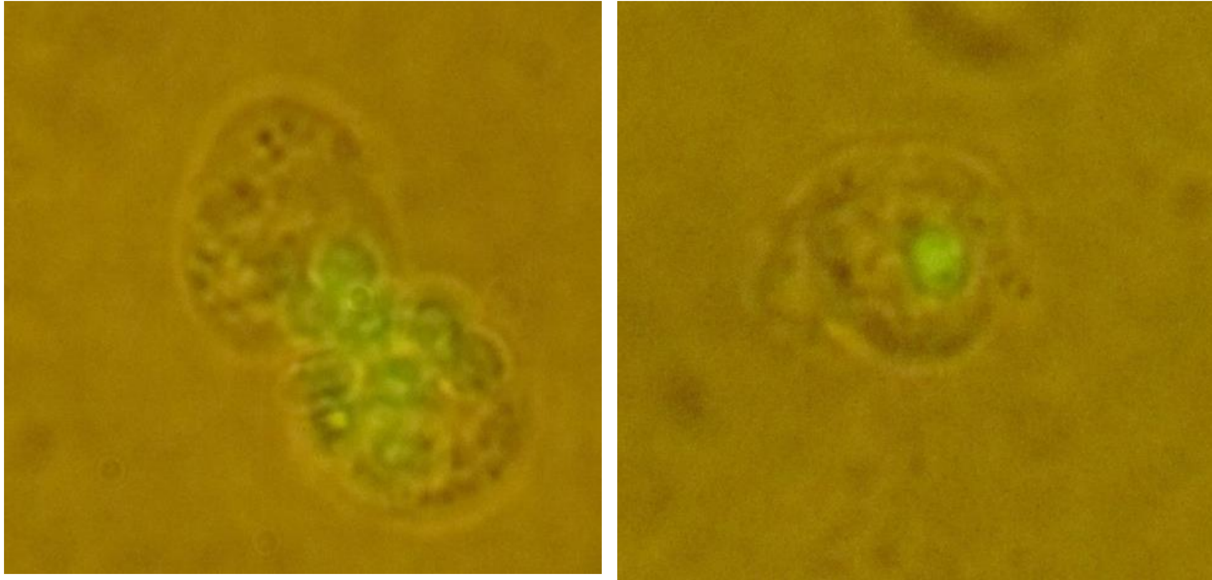


Fig. 5 Phagocytic activity, labeled conidia attach to hemocytes (100x magnification)

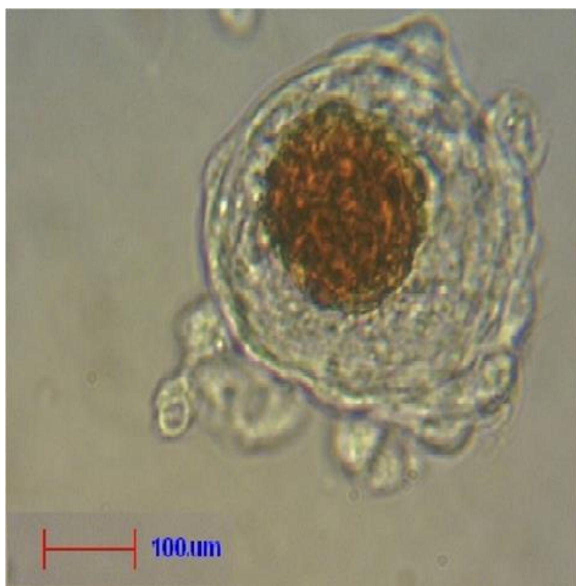
Insect cellular immune system is connected with quality and quantity of hemocytes which is different between insects. Fungal isolate, the amount of injected inocula and the experimental method may be the reasons of differences among our results with the others.

The phenoloxidase activity was different in three insects and in exposition to various fungal isolates. As, the enzyme activity reached a maximum value in 12 h for *Z. morio* and *E. integriceps* and in 24 h for *E. kuehniella.* In the most researches, the level of phenoloxidase activity was increased in first hours of fungal infection and decreased subsequently (Ajamhassani *et al*., 2013; Dubovskiy *et al*., 2013; Khosravi *et al*., 2014). High phenoloxidase activity during infection process is because of its important roles in immune reactions such as wound healing, encapsulation and nodule formation (Lokstan and Li, 1988). Zibaee *et al.* (2013) demonstrated the higher phenoloxidase activity in 6 h after injection of *B. bassiana* conidia in adult *E. integriceps*. Ezzati-Tabrizi *et al.* (2013) investigated the phenoloxidase activity of *Plodia interpunctella* and *G. mellonella* after injection of *B. bassiana* conidia, it was higher in *P. interpunctella* and reached to maximal value in 12 h after injection in both insects. The decline may be due to the immunosuppressive effect of fungal proteins or toxic metabolites.

Comparative studies about insect immune system are very rare. Bogus *et al.* (2007) demonstrated encapsulation, nodule formation and phenoloxidase activity of *G. mellonella*, *C. vicina* and *D. pini* to *C. coronatus* conidia, among them *C. vicina* was unharmed against fungal infection and had the lowest immune reactions. They expressed that other mechanisms such as antiproteolytic capacity of host hemolymph may play a role as additional safety device. While in our study *Z. morio* was resistant to fungal isolates with high level of phenoloxidase activity and nodule formation. As the immune system is costly to insects, they try to show most efficient defense at the lowest possible cost.

According to our data, there was correlation between all studied immune system reactions and the virulence of isolates for two insects, *E. kuehniella* larvae and *E. integriceps* nymphs. DE, as the least virulent isolate, highly stimulated insect immune reactions in more experiments, vice versa TV as the most virulent isolate. It's may be because of fungal metabolites, growth of entomopathogenic fungi in the hemolymph of the host is associated with the secretion of toxins (secondary metabolites) by the pathogen (Mazet *et al.,* 1994; Clarkson and Charnley, 1996; Bandani *et al.,* 2000); secondary metabolites disable several immune mechanisms allowing the fungus to overcome and then kill its host (Bandani *et al.,* 2008; Zibaee *et al.,* 2011). Different entomopathogenic fungal species and isolates have varied secondary metabolites (Sowjanya *et al.,* 2008; Mohammadi Sharif *et al.,* 2010; Molnar *et al.,* 2010) and different fungal isolates may induce immune reactions in different levels. Which may affect their host immune system reactions.

In conclusion, the immune reactions of insects showed considerable effect on susceptibility to fungal invasion. These reactions varied by host types and fungal isolates. According to our data, *E. integriceps* was the most sensitive insect with lowest immune responses against fungal infection .Conversely, *Z. morio* was resistant to all fungal isolates with high anti-fungal reactions. Obviously, further research is needed to find the other reasons and dimensions of insect resistance or susceptibility to fungal diseases.



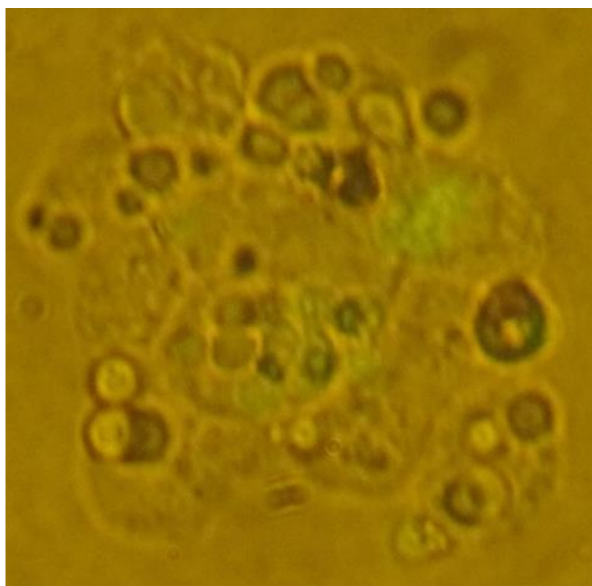


Fig. 6 Aggregation of hemocytes and entrap of labeled conidia (6 h after injection, 100x magnification) and melanization (24 h after injection, 40x magnification)

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