

Immune response of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to different entomopathogenic fungi

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Abstract

The current study reports mortality and effects on cellular immune response of several entomopathogenic fungi including isolates BB1, BB2 and BB3 of *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosoroseus* and *Lecanicilium lecanii* against larvae of *Chilo suppressalis*. Prohemocytes, granulocytes, plasmatocytes and oenocytoids were identified as the main circulating hemocytes in the hemolymph of larvae using Giemsa staining solution. Entomopathogenic fungi caused differential mortality on larvae: BB1, BB3, *M. anisopliae* lead to the highest mortality on larvae and *L. lecanii* caused the lowest mortality. The highest numbers of total hemocytes were observed 3 h post-injection of *B. bassiana* isolates and 6 h for the other treatments. The highest numbers of plasmatocytes were observed 3 h post-injection of BB1 and Tween 80, whereas BB2, BB3, *M. anisopliae*, *I. fumosoroseus* and *L. lecanii* caused plasmatocyte increase 6 h post-injection. Similar results were obtained in case of granulocytes but only Tween 80 showed the highest number of hemocytes 3 h post-injection. The highest numbers of nodules were found at various time intervals after injection of fungal isolates and latex bead. The highest activities of phenoloxidase were observed 12 h post-injection by BbB1, BbB3, *M. anisopliae* and latex bead; 3–6 h post-injection by BbB2, 6 h post-injection by *I. fumosoroseus* and 3–6 h post-injection by *L. lecanii*. Our data demonstrate the possibility of utilizing different fungal extracts in the field to help reduce the risk of resistance evolution in *C. suppressalis* and encourage experimentations aimed to increase the number of biological control agent for insect pests such as the striped rice stem borer *C. suppressalis*.

Keywords: immunity, rice striped stem borer, entomopathogenic fungi

(Accepted 9 October 2013; First published online 9 January 2014)

Introduction

Insect immune response encompasses all the processes by which insects defend themselves against invading organisms such as bacteria, fungi and parasitoids (Gillespie *et al.*, 1997;

Soderhall & Cerenius, 1998; Lavine & Strand, 2002; Schmid-Hempel, 2005; Zibae *et al.*, 2011). The recognition of a foreign particle in the hemocoel induces several cell-mediated responses like changes in hemocyte numbers, clotting, phagocytosis, nodule formation, encapsulation and melanization (Gillespie *et al.*, 1997; Schmid-Hempel, 2005). In addition of the cell-mediated immunity, humoral immunity is activated through the synthesis of antimicrobial peptides, lysozymes and activation of phenoloxidase (PO) cascade as well as the production of reactive intermediates of oxygen and nitrogen (Eleftherianos *et al.*, 2006; Haine *et al.*, 2008; Ye *et al.*, 2009; Loughton *et al.*, 2011).

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Chilo suppressalis Walker (Lepidoptera: Crambidae) is one of the major rice stem borers generating severe economic impact on rice production in Iran, Southern and East areas of Asia and South America (Zibae *et al.*, 2009). Larval stages feed on inner parts of rice stem leading to dead heart and white head as identified symptoms of damage (Khanjani, 2006). *C. suppressalis* produces two to three generations per year generating severe damage to rice plants due to intense feeding, for that reason comprehensive management strategies are needed to decrease constraints on rice production. Although there are some natural biocontrol agents in the field like the wasps *Trichogramma* spp. and the predatory bug *Andrallus spinidens* Fabricius (Zibae *et al.*, 2012). However, usually the primary method of control of the pest relies on spraying of chemical pesticides such as Diazinon (more than 95% of fields), Padan and Fenitrothion. Zibae *et al.* (2009) reported increased resistance of *C. suppressalis* in several regions of Iran, with some populations displaying up to 13.67-fold resistance to diazinon compared to susceptible populations. Esterases, alkaline phosphatases and glutathione transferase seem to be involved in developing resistance.

The combination of the severe damages generated by *C. suppressalis* and the resistance to Diazinon raises concerns about its indiscriminate use of this insecticide. Diazinon is still used in 200,000 out of 600,000 ha of rice fields with an estimated release of 4000–8000 t diazinon year⁻¹ (Vakili, 1998). In this context, the utilization of biological control agents such as entomopathogenic fungi represents a promising choice. Majidi-Shilsar (2002) demonstrated pathogenicity of *Beauveria bassiana* on all developmental stages of *C. suppressalis* egg masses, larvae, pupae and adults. Majidi-Shilsar *et al.* (2008) studied pathogenicity of *B. bassiana* on first- and second generation *C. suppressalis* larvae in field conditions. Results showed that fungus could infest 60% of larvae inside the rice stems. Pathogenicity of *B. bassiana* on *C. suppressalis* has been well documented, but there is no information on the pathogenicity exerted on *C. suppressalis* by other entomopathogenic fungi. The successful control of an insect population through entomopathogenic fungi relies on well-balanced host–pathogen–environment interactions and requires the understanding of factors responsible for insect susceptibility and resistance to a pathogen. (Hajek & St. Leger, 1994; Chouvenec *et al.*, 2009). For this reason, the objective of this study was to analyze the immune responses of *C. suppressalis* larvae to different isolated fractions of the entomopathogenic fungi *B. bassiana*, *Metarhizium anisopliae*, *Isaria fumosoroseus* and *Lecanicillium lecanii*.

Materials and methods

Insect rearing

Rice striped stem borer pupae were collected from rice fields and reared on the same variety of seedling (Hashemi). Insect were reared using Zibae *et al.* (2009) methodology with slight modifications, laboratory conditions were kept at 28 ± 1°C, 80% relative humidity (RH) and 16 h light : 8 h dark (LD 16:8). Hatched larvae fed on rice seedlings until they reached fourth instars. Laboratory conditions were checked daily, containers were cleaned and fresh stems were provided for larvae.

Entomopathogenic fungi culture

B. bassiana (Isolates: B1, B2 and B3 from Fashand, Iran), *M. anisopliae* (one isolate), *I. fumosoroseus* (one isolate) and *L. lecanii* (Isolate: LiR) were cultured at 25 ± 1°C on Sabouraud Dextrose Agar (pH=5.6) amended with 1% yeast extract. After 14 days, conidia were washed off with a 0.01% solution of Tween 80 (Sigma Aldrich, USA) and different concentrations of spores were prepared.

Determination of hemocyte types by light microscopy

Hemolymph from ten larvae of *C. suppressalis* (fourth instar) was carefully collected from one of the wounded prolegs with a 50 µl sterile glass capillary tube (Sigma Aldrich). The collected hemolymph was immediately diluted in an anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid and pH=4.6) as described by Azambuja *et al.* (1991) in 1:8 (anticoagulant/hemolymph) proportion. Hemolymph samples were directly put onto a glass slide and allowed them to dry at dark in natural air conditions for 20–30 min. Six replicates were performed to obtain images from hemocytes. Cells were then fixed in methanol for 10 min at room temperature. Fixed hemocytes were stained with Giemsa (diluted 1:9 in distilled water) for 15–35 min and slides were rapidly rinsed with distilled water (Brayner *et al.*, 2005). After staining the slides were dehydrated in ethanol and mounted in xylene. Hemocytes were monitored under light microscope (Olympus company).

Bioassay

Spores of 14-day-old SDA culture were removed by a scalpel and the serial concentrations of each Entomopathogenic fungi (EF) were prepared from 10² to 10⁸ spores ml⁻¹. Larvae were topically exposed by adding 2 µl of the solution with a sampler device (Brand Co., Germany). Control larvae were treated with 2 µl of Tween 80 (0.05%) solution. Mortality was recorded to death of individuals in the highest concentration of EFs. Lethal concentration values were calculated using POLO-PC software.

Effect of fungal spore isolates on circulating hemocyte number

To determine the effects of entomopathogenic fungi on hemocyte number, fourth instar larvae were injected laterally into the third thoracic segment with 1 µl of solution containing 10⁵ spores ml⁻¹ of the fungal isolates in Tween 80 (0.05%). Latex beads¹ and Tween 80 (0.05%) solution were used as positive and negative controls, respectively. Hemolymph was collected at intervals of 1, 3, 6, 12, 24, 48 and 72 h after injection from the control, latex bead- and spore-injected groups, separately. Samples of hemolymph from five larvae were blend in a 1.5 ml plastic tube with 1 ml of ice-cold anticoagulant buffer. The tubes were gently inverted five to seven times

¹ Ten microliters of carboxylate-modified polystyrene latex beads, 0.3 mm diameter (aqueous solution, 10% solids content, Sigma Co., St. Louis, MO, USA), were diluted 1:10 in sterile saline phosphate buffer (PBS, pH 7.2) [Borges *et al.*, 2008, *Micron* 39 486–494].

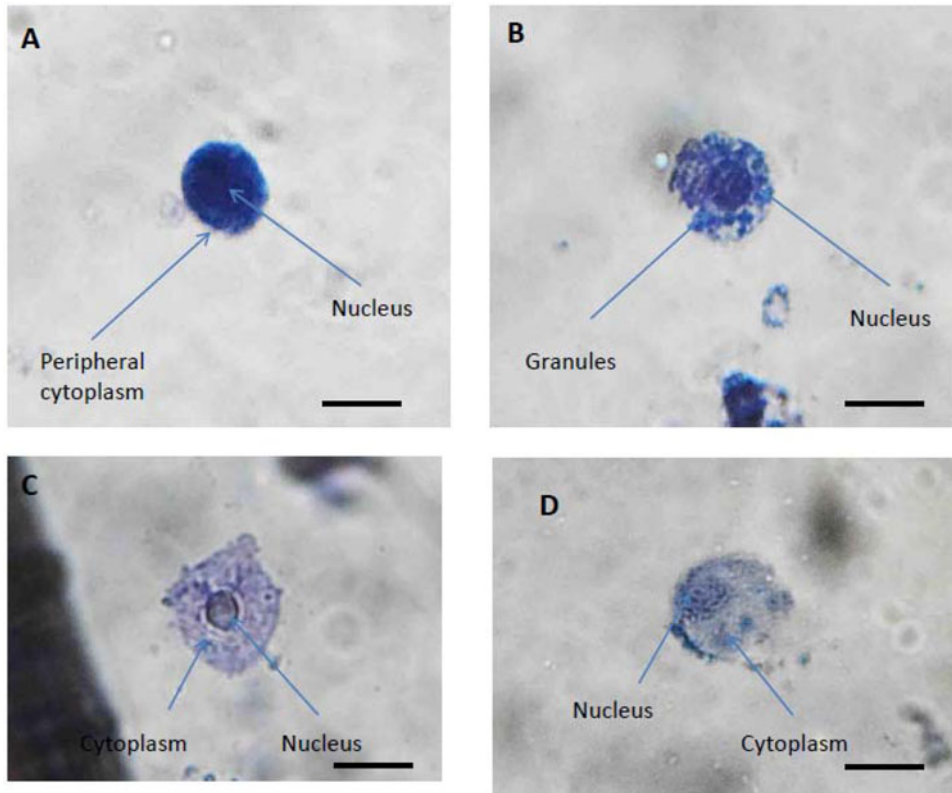


Fig. 1. Light microscopy images of *C. suppressalis* hemocytes: (A) prohemocyte with a large nucleus and a thin peripheral cytoplasm; (B) granulocyte filled with numerous granules in the cytoplasm and large nucleus; (C) plasmatocyte exhibiting a spindle shape, small nucleus and a few granules. (D) oenocytoid with a large nucleus and agranular cytoplasm. Magnification 40 \times with the exception of (b) (60 \times). Bar=5 μ m.

to facilitate mixing, and both total and specific hemocyte numbers were counted using an improved Neubauer hemocytometer (Chemkind Co.). For each treatment (Time interval), six larvae were used and the experiment had five replicates ($N=30$ for each isolate, $n=5$).

Effect of fungal spores on nodulation

After spore-injections the numbers of nodules were calculated after 1, 3, 6, 12, 24, 48 and 72h. Injected larvae were chilled on ice, hemolymph was collected in a capillary tube and 200 μ l were placed onto a hemocytometer for nodule counting (Franssens *et al.*, 2006). For each treatment (time interval), six larvae were used and the experiment had five replicates ($N=30$ for each isolate, $n=5$).

Effects of fungal spores on PO activity

After injecting the larvae with fungal spores, the hemolymph was collected after 1, 3, 6, 12, 24, 48 and 72h as mentioned earlier. The hemocyte lysate was prepared based on the protocol described by Leonard *et al.* (1985). Collected hemolymph was mixed with anticoagulant buffer and centrifuged at 13,000rpm for 5min; the supernatant was discarded and the pellet washed gently twice with a phosphate buffer (0.02M, pH=7.1). Cells were homogenized in 200 μ l of phosphate buffer centrifuged at 13,000rpm for

15 min, and the supernatant was used in PO assays. Samples (10 μ l) were pre-incubated with phosphate buffer solution at 30 $^{\circ}$ C for 3 min followed by adding of 20 μ l of a 10 mM solution of dihydroxyphenylalanin. The mixture was incubated for an additional 5 min at 30 $^{\circ}$ C and PO activity was measured in the spectrophotometer at 495 nm. One unit of PO activity represents the amount of enzyme required to produce an increase in absorbance of 0.01 units of absorbance per min (Dularay & Lackie, 1985). Activity of injected larvae was compared with that of Tween 80-injected controls ($n=3$).

Statistical analysis

Data were analyzed using one-way analysis of variance using (add procedure) (SAS reference). Tukey's studentized test was performed on those comparisons displaying significant differences ($P\leq 0.05$). To determine the differences among fungi in each time point a factorial test was performed (SAS, 1997).

Results

Determination of hemocyte types by light microscopy

Light microscopy observations after Giemsa staining of larval hemolymph revealed the presence of four morphological distinct types of hemocyte (fig. 1A–D) (Lavine & Strand, 2002). Prohemocytes are a small, oval cells with a large

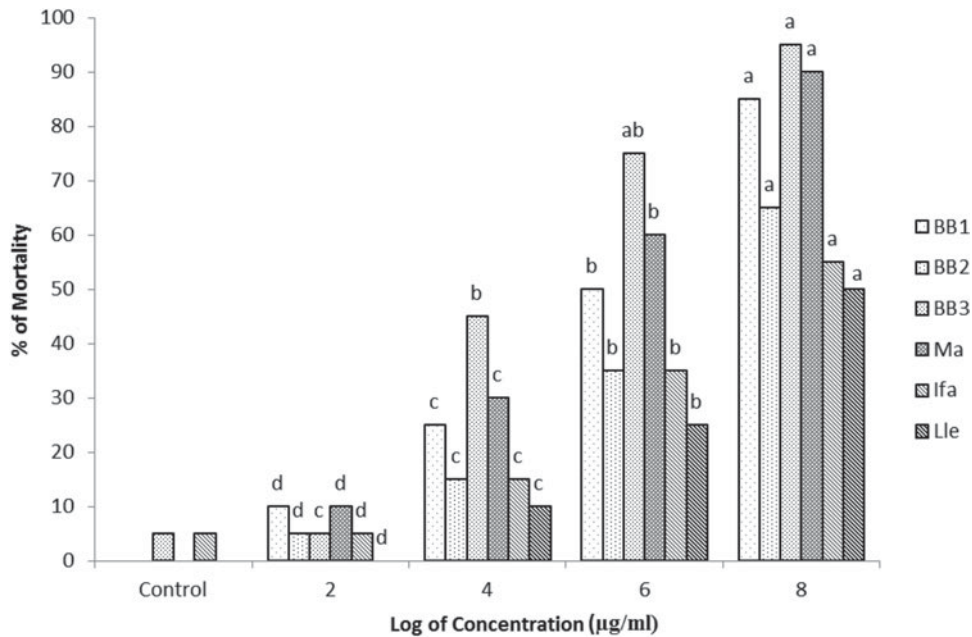


Fig. 2. Mortality of entomopathogenic fungi on larvae of *C. suppressalis*. Bars with different letters are statistically different (Tukey's test, $P \leq 0.05$).

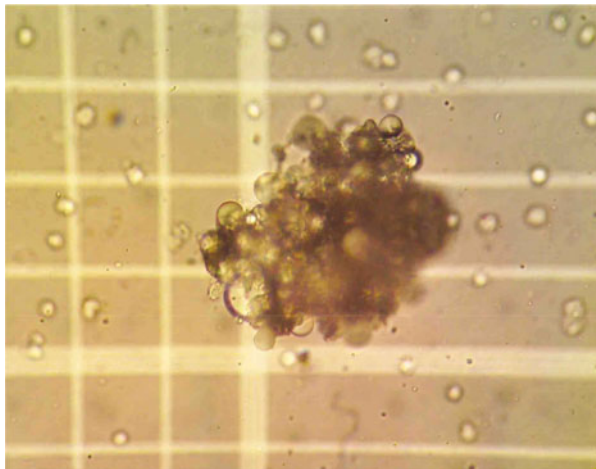


Fig. 3. Nodule formation in *C. suppressalis* 6 h after injection by different entomopathogenic fungi.

and central nucleus and thin cytoplasm (fig. 1A). Granulocytes display a regularly ellipsoidal shape, a large nucleus and a highly granular cytoplasm (fig. 1B). Plasmatocytes are large spindle-shaped cells, with central nucleus and very few granules in the cytoplasm (fig. 1C). Oenocytoids are a circular cell with a large unconventional nucleus and a granular cytoplasm (fig. 1D).

Effects of fungi on mortality of larvae

All entomopathogenic fungi used had different effects on mortality of *C. suppressalis* larvae in a dose-dependent manner (fig. 2). The highest mortality (90%) was observed at

10^8 spore ml^{-1} up to 90% (fig. 3). Specifically, BB1, BB3 and *M. anisopliae* lead to the highest mortality on larvae and *L. lecanii* caused the lowest mortality on larvae (fig. 3).

Effect of fungal spore on hemocyte numbers

Table 1 summarizes the total hemocyte counts over time in *C. suppressalis* larvae injected with spores of different entomopathogenic fungi. The highest number of total hemocytes was obtained 3 h after injection by spores of the three isolates of *B. bassiana* (table 1, $F=20.18$, $P>F: 0.001$). The highest number of total hemocytes after the injection of *I. fumosoroseus* or *M. anisopliae* spores was found 6 h post-injection, even though of the effects of *M. anisopliae* isolates after 6 h were comparable to those registered 12 h post-injection (table 1, $F=0.024$, $P>F: 2.92$; $F=0.0115$, $P>F: 3.43$). LiR isolates from *L. lecanii* and latex beads caused the highest total hemocyte numbers between 3 and 6 h post-injection (table 1, $F=3.43$, $P>F: 0.0115$).

In terms of differential count, the highest number of granulocytes was found 3 h post-injection of BbB1 and BbB3 isolates and 6 h after injection of BbB2 spores (table 2). *I. fumosoroseus* and *M. anisopliae* isolates exerted their maximal effects after 6 h, when the highest number of granulocytes was observed (table 2, $F=2.92$, $P>F: 0.0245$, $F=3.43$, $P>F: 0.0115$). Similarly, the highest number of granulocytes after *L. lecanii* spore injection and latex beads was observed 6 h after injection (table 2). For plasmatocytes, the highest number was observed after 3 h for BbB1 isolate and 6 h for BbB2 and BbB3 isolates of *B. bassiana* (table 3, $F=6.59$, $P>F: 0.0002$; $F=20.18$, $P>F: 0.0001$). For granulocytes, the number of circulating plasmatocytes reached its maximum after 6 h of spore injection (table 3, $F=2.92$, $P>F: 0.0245$; $F=3.43$, $P>F: 0.115$). After injection of LiR and latex beads, the highest number of circulating plasmatocytes was registered 6 h post-injection (table 3, $F=3.43$, $P>F: 0.0115$).

Table 1. Changes of total hemocyte numbers in *C. suppressalis* larvae injected by 10^5 spore ml^{-1} of different entomopathogenic fungal isolates.

Treatment	Time (h)						
	1	3	6	12	24	48	72
BbB1	180 ± 9.35 ^{bc*}	525 ± 27.38 ^{a*}	305 ± 96.95 ^{b*}	170 ± 18.37 ^{bc*}	165 ± 12.74 ^{bc*}	60 ± 16.95 ^c	51 ± 20.33 ^c
BbB2	160 ± 49.11 ^{bc}	340 ± 59.47 ^a	275 ± 33.54 ^{ab}	150 ± 28.50 ^{bc}	130 ± 32.97 ^b	75 ± 33.54 ^{c*}	90 ± 42.27 ^{c*}
BbB3	125 ± 15.81 ^{bc}	290 ± 42.27 ^a	235 ± 15 ^{ab}	100 ± 20.91 ^{bc}	85 ± 29.15 ^{bc}	55 ± 12.24 ^c	45 ± 22.91 ^c
If	80 ± 26.69 ^c	110 ± 21.79 ^{bc}	265 ± 45.82 ^a	160 ± 15 ^b	65 ± 26.92 ^c	80 ± 21.50 ^{c*}	25 ± 11.18 ^d
Ma	120 ± 18.37 ^b	170 ± 12.24 ^{ab}	265 ± 45.82 ^a	210 ± 12.74 ^a	105 ± 36.57 ^b	75 ± 33.54 ^{c*}	25 ± 11.18 ^d
LiR	35 ± 21.79 ^c	120 ± 37.41 ^a	130 ± 26.69 ^a	85 ± 24.49 ^{ab}	55 ± 25.49 ^b	40 ± 18.70 ^b	26 ± 15.44 ^c
Latex Beads	30 ± 12.24 ^b	145 ± 16.58 ^a	140 ± 12.74 ^a	75 ± 33.54 ^{ab}	40 ± 10 ^b	40 ± 18.70 ^b	25 ± 11.18 ^b
Tween 80	10 ± 6.12 ^b	55 ± 20 ^a	60 ± 30.20 ^a	40 ± 23.18 ^{ab}	20 ± 12.24 ^b	35 ± 21.79 ^{ab}	30 ± 14.57 ^{ab}

¹Mean ($n=5$) values must be considered as $\times 10^4$ cells ml^{-1} .

²Different letters indicate significant differences among times in each treatment ($P \leq 0.05$). Also, asterisks showed significant differences in each time intervals for all treatments (column).

Table 2. Changes in plasmacyte number in *C. suppressalis* larvae injected by 10^5 spore ml^{-1} of different entomopathogenic fungal isolates.

Treatment	Time (h)						
	1	3	6	12	24	48	72
BbB1	60 ± 6.12 ^{ab}	100 ± 15.81 ^{a*}	35 ± 10 ^b	55 ± 9.35 ^{ab}	40 ± 6.12 ^b	20 ± 9.35 ^b	30 ± 12.25 ^b
BbB2	95 ± 14.58 ^{ab*}	65 ± 12.75 ^b	105 ± 24.24 ^a	65 ± 12.75 ^{b*}	50 ± 17.68 ^b	25 ± 13.69 ^c	35 ± 21.79 ^{bc*}
BbB3	50 ± 11.18 ^b	55 ± 9.35 ^b	105 ± 24.24 ^a	55 ± 21.51 ^b	50 ± 17.68 ^b	35 ± 12.75 ^{bc*}	20 ± 9.35 ^c
If	40 ± 10 ^{ab}	55 ± 14.58 ^{ab}	70 ± 16.12 ^a	40 ± 5.63 ^{ab}	20 ± 9.35 ^b	25 ± 7.90 ^b	15 ± 3.22 ^b
Ma	45 ± 5 ^b	55 ± 12.25 ^b	125 ± 23.75 ^{a*}	65 ± 12.72 ^{b*}	85 ± 23.18 ^{ab*}	35 ± 15 ^c	35 ± 12.79 ^{c*}
LiR	30 ± 12.25 ^{ab}	30 ± 14.58 ^{ab}	70 ± 9.35 ^a	35 ± 10 ^{ab}	35 ± 6.24 ^{ab}	25 ± 15.81 ^{ab}	17 ± 4.54 ^b
Latex Beads	35 ± 2.75 ^{ab}	35 ± 2.75 ^{ab}	65 ± 12.10 ^a	30 ± 2.25 ^{ab}	25 ± 1.90 ^b	20 ± 1.58 ^b	10 ± 1.24 ^b
Tween 80	21 ± 1.86 ^a	21 ± 1.27 ^a	22 ± 1.83 ^a	25 ± 5.81 ^a	21 ± 1.86 ^a	20 ± 1.58 ^a	30 ± 1.58 ^a

¹Mean ($n=5$) values must be considered as $\times 10^4$ cells ml^{-1} .

²Different letters indicate significant differences among times in each treatment ($P \leq 0.05$). Also, asterisks showed significant differences in each time intervals for all treatments (column).

Table 3. Changes in granulocyte number in *C. suppressalis* larvae injected by 10^5 spore ml^{-1} of different entomopathogenic fungal isolates.

Treatment	Time (h)						
	1	3	6	12	24	48	72
BbB1	90 ± 15 ^{ab}	120 ± 20 ^a	35 ± 2.12 ^c	50 ± 7.90 ^b	70 ± 8.94 ^{b*}	25 ± 1.18 ^c	25 ± 1.29 ^c
BbB2	90 ± 12.75 ^{ab}	70 ± 1.58 ^b	136 ± 21.12 ^{a*}	65 ± 11.96 ^{b*}	55 ± 9.35 ^b	30 ± 1.58 ^c	20 ± 1.25 ^d
BbB3	175 ± 35.36 ^{ab*}	160 ± 12.75 ^{a*}	130 ± 16.58 ^{ab*}	55 ± 3.02 ^b	55 ± 9.35 ^b	30 ± 1.58 ^{bc}	20 ± 1.25 ^c
If	35 ± 6.12 ^b	65 ± 6.12 ^{ab}	80 ± 10.75 ^a	60 ± 6.69 ^{ab}	35 ± 1.24 ^b	15 ± 1.36 ^c	10 ± 0.68 ^c
Ma	50 ± 1.18 ^{bc}	45 ± 9.35 ^{bc}	125 ± 13.54 ^{a*}	55 ± 4.2 ^{bc}	75 ± 2.85 ^b	30 ± 1.35 ^c	30 ± 1.58 ^{c*}
LiR	30 ± 1.48 ^{ab}	30 ± 1.35 ^{ab}	45 ± 9.35 ^a	30 ± 1.58 ^{ab}	15 ± 1.12 ^b	10 ± 1.12 ^b	5 ± 1.00 ^b
Latex Beads	45 ± 5.00 ^{ab}	30 ± 5.00 ^b	85 ± 4.49 ^a	40 ± 1.75 ^{ab}	45 ± 1.35 ^{ab}	40 ± 1.75 ^{ab*}	15 ± 1.00 ^b
Tween 80	37 ± 1.94 ^a	35 ± 1.00 ^a	45 ± 4.58 ^a	35 ± 1.75 ^a	25 ± 1.90 ^b	37 ± 1.94 ^{a*}	33 ± 1.15 ^a

¹Mean ($n=5$) values must be considered as $\times 10^4$ cells ml^{-1} .

²Different letters indicate significant differences among times in each treatment ($P \leq 0.05$). Also, asterisks showed significant differences in each time intervals for all treatments (column).

Effect of fungal spores on nodulation

Injection of *C. suppressalis* larvae with spores of BbB1, BbB2 and BbB3 isolates of *B. bassiana* exerted its maximal effects on nodule formation (fig. 3) after either 12 or 24 h (table 4, $F=6.59$, $P>F: 0.0002$; $F=20.18$, $P>F: 0.0001$) (fig. 3). *I. fumosoroseus*, *M. anisopliae* and *L. lecanii* generated maximum nodule formation 12 h after the injection, but in the case of LiR, there was no significant differences between time intervals of 12 and 24 h (table 4, $F=2.91$, $P>F: 0.0245$; $F=3.43$, $P>F=0.0115$).

Latex beads injection of larvae resulted in the highest number of nodules after 6 h of the injection (table 4, $F=2.941$, $P>F: 0.0245$).

Effects of fungal spores on PO activity

The highest activities of PO after spore injection of BbB1, BbB2 and BbB3 were obtained after 12, 6 and 12 h, respectively (table 5, $F=3.43$, $P>F: 0.0115$), with the BbB3 extract showing

Table 4. Number of nodules formation counted in *C. suppressalis* larval hemolymph after injection with 10^5 spore ml⁻¹ from different entomopathogenic fungal isolates.

Treatment	Time (h)						
	1	3	6	12	24	48	72
BbB1	5 ± 0.23 ^c	5 ± 0.50 ^c	15 ± 1.00 ^b	30 ± 1.35 ^{ab}	40 ± 1.12 ^{a*}	15 ± 1.00 ^b	–
BbB2	–	10 ± 1.61 ^a ^b	35 ± 1.50 ^{a*}	31 ± 1.96 ^a	15 ± 1.12 ^{ab}	4 ± 1.87 ^b	–
BbB3	10 ± 1.12 ^{c*}	40 ± 1.75 ^{a*}	35 ± 1.50 ^{a*}	40 ± 1.00 ^a	20 ± 0.5 ^b	15 ± 1.5 ^b	–
If	–	10 ± 1.12 ^b	10 ± 1.12 ^b	40 ± 1.75 ^a	15 ± 1.00 ^b	5 ± 0.26 ^c	–
Ma	–	15 ± 1.00 ^c	35 ± 1.00 ^{b*}	50 ± 3.72 ^{a*}	30 ± 4.58 ^b	30 ± 2.25 ^{b*}	25 ± 3.69 ^{bc}
LiR	–	7 ± 1.63 ^{ab}	15 ± 1.12 ^a	17 ± 1.30 ^a	15 ± 1.12 ^a	3 ± 0.22 ^b	–
Latex Beads	–	2 ± 0.85 ^b	15 ± 1.51 ^a	7 ± 0.31 ^b	7 ± 1.12 ^b	2 ± 1.35 ^b	–
Tween 80	–	–	3 ± 0.25 ^a	2 ± 0.22 ^a	1 ± 0.036 ^a	–	–

¹Different letters show statistical differences among times in each treatment ($P \leq 0.05$). Also, asterisks showed significant differences in each time intervals for all treatments (column).

Table 5. PO specific activity (U mg⁻¹ protein) measured in *C. suppressalis* larval hemolymph after injection with 10^5 spore ml⁻¹ from different entomopathogenic fungal isolates.

Treatment	Time (h)						
	1	3	6	12	24	48	72
BbB1	1.00 ± 0.10 ^{ab}	0.89 ± 0.21 ^{ab}	0.84 ± 0.03 ^b	1.62 ± 0.11 ^a	1.14 ± 0.26 ^{ab}	0.93 ± 0.16 ^{ab}	0.59 ± 0.03 ^{b*}
BbB2	0.37 ± 0.12 ^c	2.15 ± 0.15 ^a	1.93 ± 0.14 ^{a*}	0.67 ± 0.04 ^{bc}	1.04 ± 0.04 ^b	0.48 ± 0.18 ^c	0.39 ± 0.21 ^c
BbB3	2.23 ± 0.24 ^{ab*}	2.52 ± 0.04 ^{ab*}	1.88 ± 0.24 ^{b*}	3.24 ± 0.27 ^a	1.44 ± 0.81 ^c	1.71 ± 1.32 ^{b*}	0.32 ± 0.16 ^d
If	1.06 ± 0.17 ^{ab}	0.87 ± 0.28 ^{ab}	1.38 ± 0.34 ^a	0.42 ± 0.07 ^b	0.15 ± 0.014 ^c	0.13 ± 0.06 ^c	0.10 ± 0.05 ^c
Ma	0.99 ± 0.04 ^{ab}	0.65 ± 0.11 ^b	1.06 ± 0.11 ^{ab}	1.16 ± 0.23 ^a	1.13 ± 0.12 ^{ab}	0.63 ± 0.12 ^b	0.20 ± 0.01 ^c
LiR	0.57 ± 0.18 ^b	1.45 ± 0.30 ^a	1.35 ± 0.19 ^a	0.47 ± 0.05 ^b	0.46 ± 0.13 ^b	0.25 ± 0.06 ^c	0.22 ± 0.01 ^c
Latex Bead	0.54 ± 0.26 ^c	1.35 ± 0.09 ^b	1.10 ± 0.06 ^b	2.19 ± 0.53 ^{a*}	1.28 ± 0.20 ^{b*}	0.73 ± 0.10 ^c	0.33 ± 0.16 ^d
Tween 80	0.81 ± 0.06 ^a	0.66 ± 0.34 ^a	0.65 ± 0.04 ^a	0.71 ± 0.02 ^a	0.67 ± 0.16 ^a	0.72 ± 0.23 ^a	0.61 ± 0.18 ^{a*}

¹Different letters show statistical differences among times in each treatment ($P \leq 0.05$). Also, asterisks showed significant differences in each time intervals for all treatments (column).

the highest effect. After spore injections of *I. fumosoroseus*, *M. anisopliae*, LiR and latex beads, PO activity was observed to reach its maximum between 6 and 12 h post-injection (table 5).

Discussion

The environmental concerns raised by the continued use of chemical pesticides, the rising of resistant strains of insect pests and the suitable conditions for the utilization of entomopathogenic fungi in rice fields, represent promising premises for their use as biological control agent of *C. suppressalis* in Iran. The identification of hemocyte types of an insect represents the first step of a comprehensive immune characterization and it is fundamental for understanding how the immune system may react against a specific pathogen. Through light microscopy observations, four basic types of hemocytes were identified in larvae of *C. suppressalis* prohemocytes, granulocytes, plasmatocytes and oenocytoids. These types of hemocytes have been well documented in other lepidopteran larvae (Lavine & Strand, 2002; Nakahara *et al.*, 2009). In *Bombyx mori* L. (Lepidoptera: Bombycidae) frequently referred to as the main model for lepidopteran immunity, it has been pointed out that prohemocyte is a multi-potent stem cell (Yamashita & Iwabuchi, 2001), plasmatocytes and granulocytes are responsible of cell-mediated immune reactions (Wago, 1991) and oenocytoids are involved in melanization (Iwama & Ashida, 1986). Nakahara *et al.* (2009) used various arrays of flow-cytometry to characterize

silkworm hemocytes. Also, Nakahara *et al.* (2009) synthesized cDNA from these hemocytes and subset-specific gene expression was examined by RT-PCR (Nakahara *et al.*, 2009). Results revealed that granulocytes, plasmatocytes and oenocytoids expressed different classes of immune genes and showed their multiple roles in silkworm immunity. Studies have been shown that spherulocytes has not genetically based immune functions because they failed to express most of the immune-involved genes. So, it has been suggested to play a distinct role from the other three cell types (Nakahara *et al.*, 2009). Due to the correspondence between *C. suppressalis* and *B. mori* cell morphology, we used the results collected by Nakahara *et al.* (2009) to interpret the changes of granulocytes and plasmatocytes number as a marker of the immune challenge represented by the injection of spores from different entomopathogenic fungi.

Isolates from entomopathogenic fungi modified both total and specific hemocyte number in *C. suppressalis* larvae. Although, isolates from diverse fungi exerted effects of different amplitude, in general the highest increase in hemocyte number was observed from 3 to 6 h post-injection. The increase observed within the first 24 h was followed by a decrease 72 h post-injection. This type of fluctuation in hemocyte, number of insects during pathogenic challenges have been previously recorded in *Melanoplus sanguinipes* Fabricius (Orthoptera: Acrididae) (Bidochka & Khachatourians, 1987), *Schistocerca gregaria* L. (Orthoptera: Acrididae) (Gunnarsson & Lackie, 1985), *Periplaneta americana* L. (Blattaria: Blattidae) *Spodoptera*

exigua Hubner (Lepidoptera: Noctuidae) (Hung & Boucias, 1992), *Galleria mellonella* L. (Lepidoptera, Pyralidae) (Sewify & Hashem, 2001), *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) (Chouvenec *et al.*, 2009), *Oxya japonica* Thunberg (Orthoptera: Acrididae) and *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) (Zibae *et al.*, 2011). The observed increase in total hemocyte count relies on the increment of both granulocytes and plasmatocytes, suggesting a general triggering of cell-mediated immunity against the fungal extracts. Observed decrease in hemocyte numbers at the end of the experiments is due to reduction of both granulocytes and plasmatocytes populations. Different explanations have been proposed in other insects for the trend we have observed in *C. suppressalis*: (i) a possible involvement of hemocytes in nodule formation, (ii) the cytotoxic effect of fungal secondary metabolites on hemocytes and (iii) a membrane damage due to the composition of spore surface, mainly represented by hydrophobic proteins. Mazet *et al.* (1994) reported that *B. bassiana* produced toxic metabolites while infecting the larvae of the moth *S. exigua*, thus reducing the activity of larval hemocytes. For example, Destruxins is a compound produced by *M. anisopliae* that is toxic against the hemocytes of *Manduca sexta* L. (Lepidoptera: Sphingidae) (Samuels *et al.*, 1988; Huxham *et al.*, 1989). Bandani (2005) observed that the total hemocyte number of *G. mellonella* decreased in comparison with control in a dose-dependent fashion subsequent to the infection with entomopathogenic fungus, *Tolypocladium cylindrosporum*. Zibae *et al.*, (2011) showed that fungal secondary metabolites inhibited phagocytic activity of *E. integriceps* hemocytes and hampered nodule formation. In case of latex bead and Lle, although an increase in hemocyte numbers was observed but it was so lower than *B. bassiana* isolates. This increase could be attributed to surface of spores or lower production of secondary metabolites. Meanwhile, pathogenicity of *L. lecanii* was so lower than other entomopathogenic fungi.

To explore the possible cause of hemocyte decrease, we also quantified the effects of fungal extracts on nodule formation. Nodule formation is one of the major cellular responses of insects to pathogens, especially when the invader is too big for being engulfed through phagocytosis (Chouvenec *et al.*, 2009). Our fungal extracts stimulated nodulation in *C. suppressalis* larvae the highest number of nodules was found at 6 and 12h of post-injection, in concomitance with the increase of circulating hemocytes. In these respects, the hemocyte decrease we have registered might be due to a toxic effect of fungal isolates rather than the recruitment of hemocytes in nodule formation. Solter *et al.* (1997) and Solter & Maddox (1998) proposed that infections in lepidopteran larvae were accompanied by nodule formation and melanin deposition. Gillespie *et al.* (2000) demonstrated an inverse correlation between hemocyte counts and number of nodules after a fungal infection. In our study this is not the case, and the number of nodules varied on the basis of injected entomopathogenic fungi in parallel with hemocyte number. The positive control latex beads caused the lowest nodule formation in comparison with entomopathogenic fungi. Cellular encapsulation or nodulation is induced by adhesion of hemocytes to the surface of non-self targets (Ling & Yu, 2006). Pech & Strand (1996) showed that proteins containing RGD tripeptidic motif have a critical role in encapsulation. The authors suggested that cell adhesion molecules such as integrins recognize RGD sequence and participate in

cellular encapsulation (Pech & Strand, 1995; Ruoslahti, 1996). In addition to RGD and integrins, surface characteristics of non-self targets may also be important in eliciting of encapsulation (Gorman *et al.*, 1998; Lavine & Strand, 2001). Lavine & Strand (2001) found no encapsulation of latex bead incubated by *Pseudoplusia includes* Walker (Lepidoptera: Noctuidae) hemocyte *in vitro*. Conversely, encapsulation occurred after pre-incubation of beads by plasma or injection *in vivo* and Lavine & Strand (2001) suggested the involvement of humoral recognition molecules in encapsulation.

PO cascade and melanization are intimately connected with encapsulation. POs are stocked pre-enzymes or zymogens in insect hemolymph and they become activated upon wounding or infection as part of the innate immune response (Kanost & Gorman, 2008). The enzymes have two biochemical function in hydroxylation of tyrosine to form L-dihydroxyphenylalanine, and oxidizing *o*-diphenols to form quinones (Gorman *et al.*, 2007). After forthcoming reactions, quinones form melanin, which is deposited on the surface of encapsulated parasites, hemocyte nodules and wound sites (Kanost & Gorman, 2008). In our experiments, the highest activity of PO was found after 6 and 12h post-injection which it corresponds to the hemocyte increase and nodule formation. Results may be explained by melanin deposition as a complement of the nodule formation process. The melanin deposition (melanization) of nodules and capsules around non-self targets is one of the important defensive responses in insects (Gillespie *et al.*, 1997; Christensen *et al.*, 2005; Michel & Kafatos, 2005; Nappi & Christensen, 2005; Chouvenec *et al.*, 2009; Zibae *et al.*, 2011). Melanin deposition blocks absorption of nutrients by parasites leading to their killing due to starvation (Chen & Chen, 1995). Also, formation of cytotoxic reactive oxygen and nitrogen intermediates during melanin synthesis causes to kill invading organisms (Nappi & Christensen, 2005).

In conclusion, our study demonstrated that *C. suppressalis* larvae present at least four different types of circulating hemocytes, in agreement with previous observations performed in other lepidopteran models. Our experiments evidenced a different susceptibility of *C. suppressalis* larvae to entomopathogenic fungi. In all cases the effects of the fungi involved cell-mediated immunity that is initially triggered and then strongly reduced. The immediate response of larvae to fungi infection was a significant increase of total circulating hemocytes. This increase included both granulocytes and plasmatocytes. However, after 3 days from the immune challenge, the number of circulating hemocytes was significantly lower. Consequently, the larvae showed nodulation and increased PO activity in the immediacy of the challenge, but progressively the phenomenon decreased. Further research is required in order to completely understand the effects of entomopathogenic fungi on immune system of *C. suppressalis*. These results combined with field mortality caused by entomopathogenic fungi will lead to the arsenal of bio-compatible controller for the striped rice stem borer *C. suppressalis*.

Acknowledgements

This research was supported by a grant of research from university of Guilan. The authors would like to thank Dr M. Fazeli-Dinan, H. Hoda and M. Salimi for their kind assistantship in the experiments.

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