Immunological Responses of *Sesamia cretica* to *Ferula ovina* Essential Oil

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Abstract

The current research was performed aiming to investigate the effects of *Ferula ovina* essential oil on the fourth instar larval hemogram of *Sesamia cretica*. Four main sorts of circulating hemocytes, including prohemocytes, plasmatocytes, granulocytes (GRs), and oenocytoides, were identified in the fourth instar larvae. Treatment of the larvae with the concentration of 1000 ppm of the essential oil led to an enhancement of the total hemocyte and GR count followed by a dose-dependent decrease at the concentrations of 2500 and 7000 ppm. Plasmatocyte numbers declined in all the treatments with more significant effects at increased doses. The greatest numbers of GRs, plasmatocytes, and total hemocytes were found after 48 h of treatment. The highest phenol-oxidase activity was recorded 12 h after treatment at the concentration of 2500 ppm. The highest effect on nodule formation was exerted by the concentration of 7000 ppm 12 h after treatment. The results of the present study clearly indicated that the treatment of larvae by the essential oil of *F. ovina* decreased the numbers of total and differential hemocyte counts although phenol-oxidase activity and the number of nodules showed no decline in the treated larvae. These results demonstrated that *Ferula ovina* essential oil has a significant effect on the immune ability of the studied insect and can be useful and usable for future research to practical management of this pest.

Key words: *Ferula ovina*, *Sesamia cretica*, hemogram, phenol-oxidase activity

Introduction

Chemical pesticides are currently utilized to control pests in highly infested plantations; however, unfavorable effects like destruction of ozone layer, contamination to the environment, toxicity in the organisms non-targeted at, resistance to pests, residues of pesticides, and direct toxicity in users are inevitable (Isman 2006). Plant essential oils ordinarily demonstrate a wide spectrum of activities, such as insecticidal, repellent, anti-feedant, oviposition deterrent, and growth-regulatory effects against pests. Some of the oils and their constituents are mainly used as spices and seasonings in foods and drinks, while their application in pest control would be exempt from pesticide registration.

*Sesamia cretica* Lederer (Lepidoptera: Noctuidae) may cause severe damage to maize plantations, particularly when infestation occurs shortly after the plant emergence (Semeada 1985, 1988). High-population densities of *S. cretica* have been recorded in several regions of Iran (Seraj 2001). However, no information is available on the effects of *F. ovina* essential oils on hemocytes and cellular immunity in *S. cretica*.

Insect immune system helps prevent pathogens from entering insect bodies (Nappi and Christensen 2005). Hemocytes in insect hemolymph are engaged in cellular and humoral immunity. Encapsulation, nodule formation, and phagocytosis are among cellular immune responses. On the other hand, humoral immune responses comprise the induced production of antimicrobial peptides, cell adhesion molecules, pro-phenol oxidase (pro-PO), lysozymes, and lectins (Hoffmann 2003; Kanost et al. 2004). As stocked pre-enzymes in insect hemolymph, POs are activated by infection or wounding and thus act as a part of the innate immune response (Kanost and Gorman 2008). Furthermore, recent researches have reported POs as important defense tools employed against various pathogens (Cerenius and Soderhall 2004; Khosravi et al. 2014). It has been shown that cellular response in insects is a major obstacle to the process of infection (Hoffmann 1995, 2003). Depending on the stages of physiological development, innate variability is observed among hemocytes within a species (Sanjayan et al. 1996; Beetz et al. 2008). Prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), and oenocytoides (OEs) are considered as the most common types of hemocytes. Nodulation is a cellular process whereas hemocytes recognize a foreign material and insulate it within the hemocoel (Mullen and Goldsworthy 2003).

*Ferula ovina* Boiss is an herbaceous plant of Umbrellaceae family. Other species in the genus are known to produce compounds...
with insecticidal, repellant, and anti-feedant properties. No information is available on the hemocytes and innate cellular responses of *S. cretica* against *F. ovina* essential oils. The current investigation was conducted to study the effects of *F. ovina* essential oil on the cellular immunity of these insects by assessing their total hemocyte counts (THCs), differential hemocyte counts, nodule formation, and PO activity in larval stage. So, significant effect on the immune ability of the studied insect can be used as an introduction to further studies in the field of practical management and immune response in this great pest.

### Materials and Methods

#### Rearing Insects
Pink borer larvae were collected from the maize fields of Varamin, south east of Tehran, Iran, and reared on maize seedlings at 25 ± 1 °C, a relative humidity of 65 ± 5%, and a photoperiod of 16 h light:8 h darkness (L:D 16 h:8 h). Fresh maize stems were daily supplied for egg laying. The hatched larvae were fed on the fresh stems of maize until they reached the fourth instar.

#### Extraction of the Plant Essential Oil
*Ferula* spp. are known to accumulate their secondary metabolites mostly in their roots. Hence, *F. ovina* roots were collected from Binalood mountain (north-east of Iran), dried in the shade with proper ventilation, and kept at 24 °C. For the isolation of the essential oil, the roots were extracted from 50 g of the air-dried material in 750 ml distilled water was carried out by hydrodistillation for 4 h (Negahban et al. 2007). To remove water after extraction, anhydrous sodium sulfate was utilized. The resultant oil was kept in l.5-ml micro-tubes at 4 °C.

#### Determination of Hemocyte Types
A drop of hemolymph was examined by cutting one of the prolegs of fourth instar larvae of *S. cretica*. The drop was allowed to dry in the dark at 0 °C for 10 min and stained with Giemsa (diluted 1:9 in distilled water) for 20–40 min (Brajenet al. 2005). To fix the hemocytes, the slides were immersed in a saturated lithium carbonate solution for 5 s and quickly rinsed in distilled water (Brajenet al. 2005). Then, the hemocytes were observed under a brightfield microscope.

#### The Effect of *F. ovina* Essential Oil on Number of Circulating Hemocytes
The fourth instar larvae were injected with 1 µl of each concentration (1000, 2500, and 7000 ppm) of *F. ovina* oils. Using a Hamilton syringe, the oils were applied to the mesosternum of the larvae after being diluted in acetone (Burkard, England). The controls received only 1 µl of acetone. Hemolymph was collected from the control and oil-treated larvae 3, 12, 24, and 48 h after the treatment. Immediate dilution of the collected hemolymph was done in an anticoagulant solution (0.01M EDTA, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, and pH = 4.6) as described by Azambuja et al. (1991) at 1:9 (anticoagulant: hemolymph) proportion. The total hemocyte, GR, and PL numbers were counted by using a hemocytometer. The bloods of five larvae were used in each treatment (five larvae for per concentration in each time) that each larva was used as a replicate.

#### The Effect of *F. ovina* Essential Oil on Nodule Formation
The numbers of nodules formed in the hemocoel were counted 3, 12, 24, and 48 h after treatment. The oil-treated larvae were chilled in ice, the hemolymph was collected in a micro-tube containing the anticoagulant solution, and a drop of the mixture was placed on the hemocytometer slide for counting the nodules (Franssens et al. 2006). One larva was used in each replication and the experiment was done with five replicates for each treatment.

#### The Effects of *F. ovina* Essential Oils on PO Activity
To evaluate the PO activity in the *S. cretica* larvae treated with the essential oil, the hemolymph was collected after 3, 12, 24, and 48 h as mentioned above, mixed with the anticoagulant buffer (0.01M EDTA, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, and pH = 4.6), kept overnight at -20 °C, and then centrifuged at 13,000 rpm for 15 min. The pelletized cells were homogenized in 100 µl Tris-HCl (pH7) and centrifuged at 13,000 rpm for 15 min. The supernatant containing hemocyte lysate was applied for the PO assay. The samples (25 µl) were transferred to each ELISA plate well containing 70 µl Tris-HCl buffer and pre-incubated at 30 °C for 3 min, which was followed by the addition of 40 µl of 10 mM L-DOPA solution as the substrate. The samples were incubated for an additional 5 min at 30 °C and their PO activities (AA650nm/min) were measured at 492 nm by a spectrophotometer (Leonard et al. 1985).

#### Statistical Analysis
Experiments were performed on a completely randomized design using one-way analysis of variance (ANOVA). Data normality was tested and was analyzed with variance analysis using SAS software (SAS 1996) and the means were compared by Tukey’s test at 5% level.

### Results

#### Determination of Hemocyte Types
Brightfield microscopy observations of the stained larval hemolymph revealed the presence of four morphologically distinct types of hemocytes. The smallest rounded cells with a large central nucleus and thin cytoplasm (*Fig. 1a*), large cells with an irregular shape and a central nucleus (*Fig. 1b*), oval-shaped ones with a large nucleus and highly granular cytoplasm (*Fig. 1c*), and circular cells with a large peripheral nucleus (*Fig. 1d*) were identified as PRs, PLs, GRs, and OEs, respectively.

#### The Effect of *F. ovina* Essential Oil on the Hemocyte Counts
Treatment of the fourth instar larvae of *S. cretica* with the different concentrations of the essential oil resulted a significant different in the number of hemocytes. THC was variably affected by the essential oil after treatments following a decrease until 12 h and an increase by 24–48 h. The highest number of THC was observed after 48 h (*Fig. 2, F = 7.14, df = 3, and P < 0.0001*). Similar results obtained for GRs and GRs showed increasing at the concentration of 1000 ppm and decreased at the concentrations of 2500 and 7000 ppm. In terms of differential hemocyte count, the highest number of GRs was counted 48 h after the treatment (*Fig. 3a, F = 4.06, df = 3, and P < 0.0001*). Also, a dose-dependent reduction in the PL numbers was observed. The treatment with the essential oil exerted
its maximal effect on PLs after 48 h (Fig. 3b, $F = 9$, $df = 3$, and $P < 0.0001$). Plasmatocytes and GRs are the important hemocytes in cellular immune responses to pathogens (Strand 2008). So at the present study, the number of these cells has been evaluated and reported.

The Effect of *F. ovina* Essential Oil on Nodule Formation

Treatment of the larvae with the essential oil exerted its maximal effect on nodule formation at a concentration of 2500 ppm and 12 h after the treatment (Fig. 4, $F = 174.25$, $df = 3$, and $P < 0.0001$). These findings are correspondence with the lower number of PLs and GRs after 12 h injection with concentration of 2500 ppm and after 24 h injection with a concentration of 7000 ppm. Hence, it could be concluded that higher production of nodules is due to the involvement of these hemocytes in nodule formation.

PO Activity

The highest PO activity was observed after treatment with the essential oil at a concentration of 7000 ppm and 12 h after the treatment (Fig. 5, $F = 20$, $df = 3$, and $P < 0.0001$). Results of the current study are carried out to melanin deposition to complete of nodule formation process.

Discussion

The study of the fourth instar larvae of *S. cretica* revealed the presence of four hemocyte types, namely PRs, PLs, GRs, and OEs, all of which were easily distinguishable in the smears using Giemsa staining. Similar results were found by Zibaee and Malagoli (2014) on the *Chilo suppressalis*, Giglio et al. (2008) on the *Carabus lefebvrei*, and Nakahara et al. (2009) on the *Bombyx mori* where brightfield microscopy demonstrated the presence of four hemocyte types (prohemocytes, PLs, OEs, and GRs). Composition of hemocyte population might differ depending on the developmental stages and insect taxa (Ribeiro and Brehelin 2006; Giglio et al. 2008). As reported, the most abundant hemocyte type in Lepidoptera larvae has been GR (Strand 2008). It has been shown that GRs, PLs, and OEs play multiple roles in silkworm immunity (Nakahara et al. 2009). GRs, PLs, and OEs are engaged in phagocytosis, nodule formation, and PO activity, respectively. The use of the plant essential oil was conducted to determine its effect on hemocytes and defense responses of
S. cretica larvae. In this study, the THC, GRs, and PLs during treatment initially increased, then decreased with increasing concentration. Reductions in THC, PLs and GRs of insects during essential oil treatment initially increased, then decreased with increasing concentration initially increased, then decreased with increasing concentration. These fluctuations could be attributed to taking part of hemocytes in nodule formation after treatment. The highest numbers of nodules were observed at a concentration of 2500 ppm and 12 h after treatment. There is no efficient study on the effect of essential oils on insects. However, different results have been showed by Zibaee and Bandani (2010) that A. annua extract decreased nodule formation in Eurygaster integriceps. Our study shows the negative effect of highest concentration of F. ovina essential oil on phenoloxidase activity of S. cretica. Similar results have been showed by Zibaee and Bandani (2010) that increasing of concentration of A. annua extract has reduction effect on the PO activity in E. integriceps. These results are carried out to melanin deposition to complete nodule formation process. Insect defenses against external agents through two cellular and humoral pathways (Schmid 2005). Several studies indicated that entrance of an invader in insect hemocoel induced two pathways and led to phagocytosis, nodule formation, encapsulation, and synthesis of antimicrobial peptides (Beckage 2008; Borges et al. 2008), Nappi and Christensen (2005) reported humoral responses including production of proPO cascade and antibacterial peptide induced after nodule formation for killing of exogenous agents. The present study shows increasing of nodule formation followed with the enhancement of PO. According to the past study, it is anticipatable that encapsulation and antibacterial peptides will induce after nodule formation and PO, although this needs more investigations. These results are not sufficient to increase the insect immune response against pathogenic microbes due to the immune system of insects that is very wide and immune responses also vary depending on the type of invader. The results of this study can serve as an introduction to the immune system S. cretica used by other researchers. It is concluded that the botanical treatment used in this study can alter the immunological responses of insect species, thus providing a promising candidate for the physiological control of S. cretica.

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