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THE SUBLETHAL EFFECTS OF FIVE INSECTICIDES ON *HABROBRACON HEBETOR* SAY PARASITISM ON *EPHESTIA KUEHNIELLA* ZELLER LARVAE BASED ON PHENOLOXIDASE ENZYME ACTIVITY

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Abstract

Ephestia kuehniella is an important cereal pest and *Habrobracon hebetor* is its efficient biocontrol control worldwide. The enzyme phenoloxidase is one of the major components of the insect immune system. This enzyme catalyzes the quinones and other reactive intermediates to reduce parasitoid invasion. In this study, the effects of five commercial insecticides in Iran on the parasitism percentage of *H. hebetor* and on the activation of phenoloxidase in the hemolymph of host larvae as an important indicator were investigated. The results showed that the percentage of parasitism was significantly lower in insecticide treatments compared to the control ($F_{5, 42}$= 6.471). There were significant differences in total protein content among the control and insecticides treatments, as well as in the phenoloxidase activity in the host larvae. Accordingly, the highest activity was in the control (0.3924±0092 µM/min/mg protein) and the lowest when larvae were treated with propargite (0.1927±0.0169 µM/min/mg protein). The results confirmed that the insecticides palizin and buprofezin do not have serious negative effects on *H. hebetor* parasitism when combined in integrated pest management.

KEY WORDS: mill moth, insecticides, natural enemy, parasitism, phenoloxidase

Introduction

The enzyme phenoloxidase is a major component of the natural defenses of invertebrates and causes melanization of pathogens and damaged tissue. The process of melanization depends on the activation of this enzyme, which is controlled by the prophenoloxidase activation system (Taleh *et al*., 2014). During prophenoloxidase activation, many other immune responses are elicited, such as cytotoxic, opsonic and encapsulating activities. The enzyme phenoloxidase plays a key role, especially in insects with three

physiologically important aspects: immune responses, cuticle sclerotization and wound healing (Chase *et al*., 2000; Cerenius & Soderhall, 2004; Narayan, 2004).

Habrobracon hebetor Say (Hymenoptera: Braconidae) (Fig. 1A) is a well-known idiobiont and ectoparasitic wasp with rapid foraging ability and compatibility with a wide range of hosts. This useful agent can decrease high populations (near economic injury level (EIL) or even higher) of moth larvae, especially those belonging to Pyralidae and Noctuidae, on different crops worldwide (Magro & Parra, 2001; Darwish *et al*., 2003; Desneux *et al*., 2007; Dweck *et al*., 2008). To date, this important biological control against invasive Lepidoptera larvae such as *Helicoverpa*, *Sesamia*, *Ostrinia* and other species has been used in many regions of Iran and the other countries worldwide (Yu *et al*., 2002; Asadi *et al*., 2018, 2019, 2020, 2021, 2022). The Mediterranean flour moth or mill moth (*Ephestia kuehniella* Zeller) (Fig. 1B) is an invasive moth of the family Pyralidae. It is a widespread pest of various cereals and processed grains, especially flour (Sarmadi *et al*., 2010). This moth is distributed worldwide, especially in countries with a temperate climate. It prefers warm temperatures to develop faster but can survive in a wide range of temperatures. This pest is commonly found in warm places on stored grain products such as mills and bakeries, where it can breed throughout the year (Navaei *et al*., 2002).

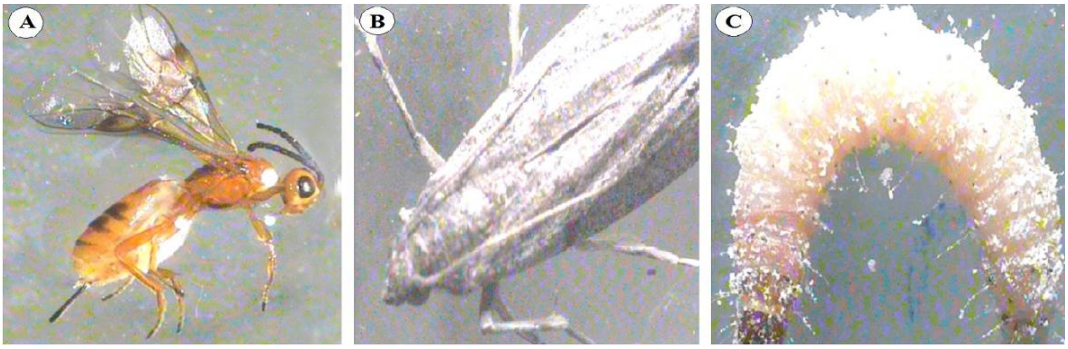

Figure 1. A – Female of *Habrobracon hebetor* Say wasp; B – female moth; and C – larva of *Ephestia kuehniella* Zeller.

Phenoloxidase is produced under various adverse conditions, including habitat stress, infection by microbial and pathogenic agents, pollution by pesticides, and even attack by biological control agents, especially parasitoids which are often paralyzed by it (Taleh *et al*., 2014). Therefore, it is reasonable to expect an increase in the production of this enzyme in larvae under parasitism by the idiobiont ectoparasitoid *H. hebetor*. Thus, this enzyme represents a suitable index for parasitism success. In other words, if an external factor such as various pesticides affects biocontrol agents, their parasitism would naturally decrease and consequently the activity of phenoloxidase in their hosts would also decrease.

To date, very limited studies have been performed on how the parasitism by *H. hebetor* affects the activity of phenoloxidase in its host (Hartzer *et al*., 2005; Kryukova *et al*., 2007, 2011; Altuntas & Kilic, 2010; Karimzadeh & Sayyed, 2011; Shehzad *et al*., 2019); there is no study on the effects of insecticides in this case. For this reason, we examined, for the first time, changes in this enzyme in the immune system of *E. kuehniella* as the laboratory host of the parasitoid wasp *H. hebetor* under treatments with five commercial insecticides. The results of this study could be useful for effective mass rearing programs of this biocontrol agent on lepidopteran pest larvae.

Materials and Methods

Insecticides

Five formulations of insecticide used in Iran (fenvalerate, propargite, dayabon, palizin and buprofezin) were used in the phenoloxidase enzyme assays on *H. hebetor*. Information about the insecticides is presented in Table I.

Table I. The selected insecticides for phenoloxidase enzyme study on *E. kuehniella* larvae.

Insecticide	Trade name (a.i.%)	Manufacturer
Fenvalerate	Sumicidin® EC (20%)	Aria Shimi (Iran)
Propargite	Omite® EC (54%)	Aria Shimi (Iran)
Dayabon	Dayabon® SL (10%)	Nano Fanavaran Daya (Iran)
Palizin	Palizin® SL (60%)	Kimia Sabzavar (Iran)
Buprofezin	Applaud® SC (40%)	Nihon Nohyaku (Japan)

EC: Emulsifiable Concentrate, SL: Soluble Liquid, SC: Suspension Concentrate.

Rearing of the host pest (*Ephestia kuehniella*)

The eggs of the mill moth (*E. kuehniella*) were provided by a private insectary (registered name: Jalilian) in Homeil, Kermanshah province, western Iran, in 2019. Then 0.3 g of the eggs were mixed with flour and bran (800 g + 200 g) under laboratory conditions (23±2°C, 50±5% relative humidity and complete darkness) (Abedi *et al*., 2012, 2014; Mahdavi & Saber, 2013). The fifth instar larvae (Fig. 1C) were used in parasitism and enzymatic studies.

Rearing of the parasitic wasp (*H. hebetor*)

Adult *H. hebetor* wasps were obtained from a private insectary (registered name: Jalilian) in Homeil, Kermanshah province, Iran, in 2019. Wasps were brought to the laboratory and reared on the last larvae of *E. kuehniella* under the conditions of 23±2 °C, 50±5% relative humidity and a 16:8 h (L:D) photoperiod. Honey was used as food for the wasps during rearing (Rafiee-Dastjerdi *et al*., 2008; Abedi *et al*., 2012, 2014).

Determination of parasitism percentage

To determine the parasitism percentage of *H. hebetor* on its host, LC_{30}, a sublethal concentration of the selected insecticides, was used as follows: 0.14 mg a.i./L for fenvalerate, 7.01 mg a.i./L for propargite, 3.04 mg a.i./L for dayabon, 9.58 mg a.i./L for palizin and 2.53 mg a.i./L for buprofezin. The surface of 10-cm Petri dishes were covered with LC_{30} of each insecticide and 100 mated emerged females of *H. hebetor* were released inside them. In the control, distilled water was used. The Petri dishes were moved to the laboratory and kept for 24 h at 23±2 °C, 50±5 % relative humidity and a 16:8 (L: D) h photoperiod. After this time, eight live females were randomly selected and introduced individually to Petri dishes containing 16 *E. kuehniella* fifth instar larvae under the above-mentioned conditions for 24 h. Each larval density was conducted under eight replicates. During the experiments, the wasps were supplied with honey, and after 24h, the number of larvae parasitized by the treated wasps were

recorded in each test unit (Abedi *et al*., 2012; Mahdavi & Saber, 2013). The experiment was carried out in eight replicates.

Determination of total protein content

The total protein content in *E. kuehniella* hemolymph samples was measured using the earlier method of Bradford (1976) and bovine serum albumin as a standard. For this purpose, concentrations of 0, 1, 2, and 4 mg/ml were prepared from the serum and 5 µl of this solution was mixed with the Bradford reagent (200 µl), distilled water (795 µl) and the solution containing the enzyme (5 µl). The absorbance of the samples was then measured at 595 nm using a spectrophotometer. Based on the available information about the protein concentration in the standard solution, a graph was plotted and the total protein was determined based on the graph information and absorbance values.

Phenoloxidase enzyme assay

The larvae of *E. kuehniella* paralyzed by the parasitoid wasp were used to measure the enzyme phenoloxidase in an assay. The larvae were first maintained in a freezer to anesthetize them, and their last prolegs were cut off with a sterile scalpel. The hemolymph was collected in 1.5-mL Eppendorf tubes containing ethylenediaminetetraacetic (EDTA) acid (0.01 M), glucose (0.01 M), NaCl (0.062 M) and citric acid (0.026 M) as anticoagulant (Fig. 2), then centrifuged (4°C, 12,000 rpm, 12 min). After these steps, the supernatant was discarded and the pellet (containing the blood cells) was washed with phosphate buffer and 0.5 mL of the buffer was added, homogenized and centrifuged again (4°C, 12 min, 12,000 rpm) (Fig. 3). Finally, 50 µl of 10 mM dihydroxy phenylalanine (L-DOPA) was added as an enzyme substrate and after incubation at room temperature for 30 min, the absorbance at 490 nm was determined by spectrophotometer. In this study, all experiments were performed in three replicates (Azambuja *et al*., 1991).

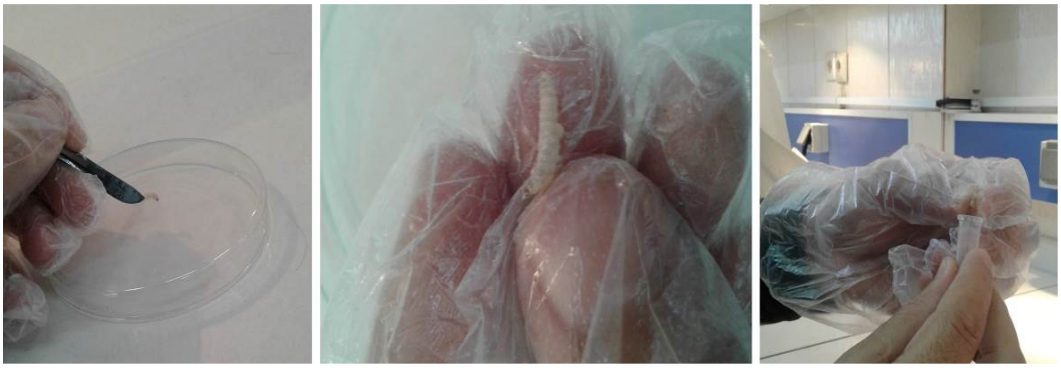

Figure 2. Isolation of hemolymph from larvae of *E. kuehniella.*

Statistical analysis

The data were checked for normality and analyzed by one-way ANOVA, using Tukey's test under the probability level of 5% in SPSS ver. 16 software.

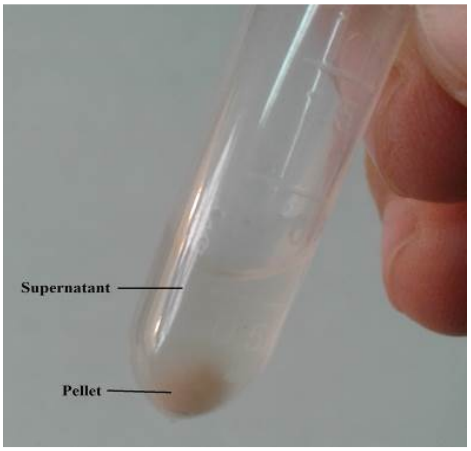

Fig. 3 Supernatant and pellet from the hemolymph of *E. kuehniella* larvae

Results

Parasitism percentage of *E. kuehniella* larvae by *H. hebetor*

The parasitism percentages of *E. kuehniella* larvae by *H. hebetor* wasps are given in Table II. Based on ANOVA, significant differences were observed between the control and the insecticide treatments ($F_{5,42}$= 6.471); however, the difference between propargite and buprofezin was not significant. In addition, the highest parasitism was seen in the control (85.93±4.53%) and the lowest in the fenvalerate treatment (58.59±3.90%). The decreasing trend was as follows: control, palizin, dayabon, buprofezin, propargite and fenvalerate.

Table II. Parasitism of *E. kuehniella* larvae by *H. hebetor* treated with insecticides and the control.

Treatment	Parasitism (%)
Control	85.93±4.53 ^{a}
Fenvalerate	58.59±3.90 ^{c}
Propargite	63.28±2.75 ^{bc}
Dayabon	71.09±3.90 ^{abc}
Palizin	78.12±4.75 ^{ab}
Buprofezin	70.31±3.28 ^{bc}

The different letters above values show significant differences (Tukey's test, P<0.05)

Total protein content in the hemolymph of *E. kuehniella* larvae

Total protein content in the hemolymph of *E. kuehniella* larvae is shown in Table III. Based on ANOVA, significant differences were found between the control and all insecticide treatments ($F_{5,12}$= 3.795).

Differences among fenvalerate, propargite and dayabon and between palizin and buprofezin were not significant. Moreover, the highest protein concentration was observed in the control (1.0288±0.0212 mg/mL) and the lowest in the palizin treatment (0.8294±0.0078 mg/mL). The decreasing trend observed was as follows: control, propargite, dayabon, fenvalerate, buprofezin and palizin.

Table III. Total protein content in the hemolymph of *E. kuehniella* larvae under the parasitism of *H. hebetor* treated with insecticides and the control.

Treatment	Protein concentration (mg/ml)
Control	1.0288±0.0212 [a]
Fenvalerate	0.8921±0.0462 [ab]
Propargite	0.9169±0.0257 [ab]
Dayabon	0.9040±0.0152 [ab]
Palizin	0.8294±0.0078 [b]
Buprofezin	0.8418±0.0668 [b]

The different letters above values show significant differences (Tukey's test, P<0.05)

Phenoloxidase activity in the hemolymph of *E. kuehniella* larvae

The results on phenoloxidase activity in the hemolymph of *E. kuehniella* larvae are shown in Table IV. Based on ANOVA, there were significant differences between the insecticides and the control ($F_{5,12}$= 34.781). The highest activity was in the control (0.3924±0092 µM/min/mg protein) and the lowest in the propargite treatment (0.1927±0.0169 µM/min/mg protein). The among between control, palizin and buprofezin and between fenvalerate and dayabon were not significant. The overall downward trend of the treatments was as follows: control, buprofezin, palizin, dayabon, fenvalerate and propargite.

Table IV. Phenoloxidase activity in the hemolymph of *E. kuehniella* larvae under parasitism of *H. hebetor* treated with insecticides and the control.

Treatment	Phenoloxidase activity (µM/ min/ mg protein)
Control	0.3924±0.0292 [a]
Fenvalerate	0.2881±0.0078 [b]
Propargite	0.1927±0.0169 [c]
Dayabon	0.2909±0.0141 [b]
Palizin	0.3529±0.0123 [a]
Buprofezin	0.3738±0.0076 [a]

The different letters above values show significant differences (Tukey's test, P<0.05)

Discussion

Our study confirmed that the sublethal effects (LC_{30}) of investigated insecticides on *H. hebetor* (including fenvalerate and propargite) greatly reduced parasitism activity. Since adequate parasitism is directly related to the activation of phenoloxidase, lower activity was naturally observed in the treatments. Conversely, treatments with lower sublethal toxicity against the parasitoid wasp (palizin and buprofezin) caused higher activity of the enzyme due to effective parasitism.

To date, few studies have been conducted on the effects of parasitism by the ectoparasitoid wasp *H. hebetor* on the phenoloxidase activity of its host larvae. Hartzer *et al*. (2005) examined phenoloxidase activity in the hemolymph of larvae parasitized by this biocontrol agent and reported that parasitism initially increased the levels of the enzyme in larval hemolymph. They related this increase to viral infection caused by the parasitism but observed that phenoloxidase activity in the hemolymph decreased dramatically with increasing parasitism. As mentioned earlier, the phenoloxidase activity is high during parasitic infestation and microbial infection, which is a natural insect response to these conditions. In another study, Kryukova *et al*. (2007) examined the immune response of *Galleria mellonella* L. parasitized by *H. hebetor* and concluded that the parasitoid wasp inhibits phenoloxidase in the hemolymph of its host. Kryukova *et al*. (2011) also investigated the effects of *H. hebetor* parasitism on phenoloxidase activity, reactive oxygen species (ROS) production and foreign matter encapsulation in the hemolymph of *G. mellonella* larvae and found that phenoloxidase activity and hemocyte numbers were greatly decreased in this pest. Additionally, they indicated that melanization of the capsules in the first larval stage was lower than in control larvae. Since phenoloxidase plays an important role in the immune system of insects, it is expected that when the activity of this enzyme is high, the resistance of insects to various factors will also increase. Therefore, with lower activity of this enzyme or its inhibition by various agents, we should expect lower resistance to various chemical and biological agents.

In another study, Altuntas and Kilic (2010) investigated the effects of parasitism by *H. hebetor* on the hemolymph proteins of *E. kuehniella* larvae and found that the amount of plasma proteins in the hemolymph slowly decreased between 24 h and 48 h after parasitization; however, their analysis showed that the amount of twelve proteins decreased, while five proteins increased in the hemolymph after mentioned times. Thus, they concluded that host regulation of *E. kuehniella* by *H. hebetor* only involves quantitative changes in plasma proteins and does not result in the production of new proteins. As mentioned earlier, any treatment of insects can alter their total protein content, which directly affects the activity of phenoloxidase. Karimzadeh and Sayyed (2011) studied the effects of interactions between the parasitoid wasp *Cotesia plutellae* (Kurdjumov) and *Bacillus thuringiensis* Berlinger on changes in the phenoloxidase of two sensitive and tolerant populations of cabbage moth and found that phenoloxidase activity slowly reduced under treatment with *B thuringiensis* and the parasitoid wasp alone; however, a combination of the two greatly reduced the activity of this enzyme in the pest larvae. Their results confirmed that the simultaneous application of *B thuringiensis* and the parasitoid wasp could have a better effect in controlling this important pest. Shehzad *et al*. (2019) studied the biochemical characteristics and phenoloxidase in the hemolymph of *G. mellonella* parasitized by *H. hebetor* and concluded that wounds and infiltration by various parasitic agents can cause the activation of immune responses in the parasitized host; therefore, the parasitoid can inhibit the mechanism of phenoloxidase production by various methods. The combination of these factors leads to the destruction of immune cells. Finally, they found that a decrease in phenoloxidase activity could be caused by a stronger effect of toxins injected by the parasitoid wasps into their host insects.

Unfortunately, so far there have been no studies on the direct effects of various chemical compounds on changes in phenoloxidase in hosts of *H. hebetor*. Thus, the present study was a new topic and should be continued on other natural enemies of their hosts. The results of this study showed that the insecticides that had more negative effects on this parasitoid wasp, decreased its parasitism activity and consequently caused

low enzyme activity; conversely, less toxic treatments caused high enzyme activity in host larvae because they had fewer negative effects.

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СУБЛЕТАЛНИ ЕФЕКАТ ПЕТ ИНСЕКТИЦИДА НА ПАРАЗИТИЗАМ *HABROBRACON HEBETOR* SAY НА ЛАРВАМА *EPHESTIA KUEHNIELLA* ZELLER НА ОСНОВУ АКТИВНОСТИ ЕНЗИМА ФЕНОЛОКСИДАЗЕ

МОХАМАД АСАДИ

Извод

Ephestia kuehniella је важна штеточина житарица, а њен природни непријатељ је *Habrobracon hebetor*. Ензим фенолоксидаза је једна од главних компоненти имуног система инсеката. Овај ензим катализује хиноне и друге реактивне интермедијере како би смањио инвазију паразита. У овој студији су испитивани ефекти пет комерцијалних инсектицида у Ирану на проценат паразитизма *H. hebetor* и на активацију фенолоксидазе у хемолимфи ларви домаћина као важног индикатора. Резултати су показали да је проценат паразитизма био значајно мањи код третирања инсектицидима у односу на контролу ($F_{5,42}$= 6.471). Постојале су значајне разлике у садржају укупног протеина између контролних и инсектицидних третмана, као и у активности фенолоксидазе у ларвма домаћина. Сходно томе, највећа активност је била у контроли (0,3924±0092 μM/min/mg протеина), а најмања када су ларве третиране пропаргитом (0,1927±0,0169 μM/min/mg протеина). Резултати су потврдили да инсектициди пализин и бупрофезин немају озбиљних негативних ефеката на паразитизам *H. hebetor* када се комбинују у интегрисаном сузбијању штеточина.

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