Acta Entomologica Serbica, 2022, 27(2): 55-65

UDC: 632.937.3
632.752:595.786
DOI: 10.5281/zenodo.7427630

THE SUBLETHAL EFFECTS OF FIVE COMMERCIAL INSECTICIDES ON THE AMYLOLYTIC AND PROTEOLYTIC ACTIVITY OF THE BIOCONTROL AGENT, *HABROBRACON HEBETOR* SAY (HYMENOPTERA: BRACONIDAE)

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Abstract

Habrobracon hebetor Say is an important biological control agent that has a wide range of lepidopteran hosts, especially from the Noctuidae and Pyralidae families. Nutrition is a very important element in the efficiency of natural enemies in integrated pest management (IPM). In the presented research, the sublethal effects of five selected chemical and botanical insecticides, fenvalerate, propargite, buprofezin, dayabon and palizin, on the activity of amylase and protease enzymes in the midgut of this important ectoparasitoid wasp were studied under laboratory conditions. Changes in the activity of enzymes were investigated by LC_{30} treatment of the female wasps, which were 0.14, 7.01, 3.04, 9.58 and 2.53 mg a.i./mL, respectively. The obtained results showed that this parasitoid wasp has a long midgut in its digestive tract. Also, there were significant differences among the enzymatic treatments ($F_{5,12}$ =14.695 and 99.278) except protein concentration ($F_{5,12}$ = 0.670). Regarding the amylase enzyme, the highest and lowest activities were obtained in the control and fenvalerate treatment (0.0435±0.0023 mU/mg vs. 0.0277±0.0010 mU/mg). In addition, the highest proteolytic activity was observed in the control (4.9817±0.0268 U/mg) and the lowest in the propargite treatment (3.2231±0.0917 U/mg). By closely investigating the results, dayabon and palizin showed the lowest negative effects on the enzymes and could be applied together with this parasitoid wasp in IPM designs.

KEY WORDS: parasitoid wasp, biological control, amylase, protease, enzyme activity

Introduction

Braconidae is a family of ecto- and endoparasitoid wasps that are closely related to the family Ichneumonidae. Braconids represent the second largest family among the order Hymenoptera with about 17,000 recognized species, many thousands of which are undescribed (Keever *et al*., 1985). One analysis estimated a total between 30,000 to 50,000 and another gave a narrower estimate between 42,000 to 43,000 species (Youm & Gilstrap, 1993; Magro & Parra, 2001). Both families play very effective roles in biological control programs, especially on the immature stages of Pyralidae and Noctuidae moths (Faal Mohammad-Ali *et al*., 2014; Asadi *et al*., 2018, 2019, 2020, 2021).

Habrobracon hebetor Say is a minute wasp belonging to the family Braconidae. It is an effective ectoparasitoid of several moth species (Paust *et al*., 2006). Well-known hosts include the larval stage of *Plodia interpunctella* Hübner (the Indian meal moth) and *Ephestia kuehniella* Zeller (the Mediterranean flour moth) (Croft, 1990; Magro & Parra, 2001; Desneux *et al*., 2007). As a cosmopolitan insect, *H. hebetor* has been used commercially by different insectariums as a natural enemy in the control of many lepidopteran pests on different agricultural and horticultural crops worldwide (Keever *et al*., 1985).

Alpha-amylase is an enzyme that catalyzes the hydrolysis of starch into sugars (Hori & Watanabe, 1980). Many insects, especially their larvae, feed on polysaccharide-rich sources and therefore make extensive use of these enzymes to break the polysaccharide chains (Valencia *et al*., 2008). Foods that are rich in starch but low in sugar, such as rice and potato, may acquire a slightly sweet taste when chewed because amylase degrades some of their starch into sugar (Stroble *et al*., 1998; Franco *et al*., 2000). The food sources used by insects and other invertebrates have significant effects on the activity of their amylases (Slansky & Scriber, 1981). Proteases are enzymes that increase the rate of proteolysis, which is the breakdown of proteins into smaller polypeptides or single amino acids (Telang *et al*., 2005). They do this by cleaving the peptide bonds within proteins by hydrolysis, which is a reaction where water breaks bonds. Proteases are involved in many biological functions including digestion of ingested proteins, protein catabolism, breakdown of old proteins, and cell signaling. Without additional assisting mechanisms, such as an enzyme or other catalyst to catalyze, proteolysis would be very slow, taking hundreds of years (Jongsma *et al*., 1995).

Biocontrol has been considered as one of the major methods in IPM (Dent, 1995; Banks and Stark, 1998). Biological control agents including parasitoids, predators and pathogens, can usually be combined with appropriate chemical and botanical compounds (Galvan *et al*., 2005; Haseeb *et al*., 2005; Mahdavi, 2013). Consequently, the monitoring and selection of suitable compounds for these sensitive agents are very important aspects of IPM. One of the obvious effects of insecticides is their digestive or stomach activity which this feature about some pesticides is very higher when reduce the activity of plant pests caused by low or no production of digestive enzymes (Parsia Aref and Valizadegan, 2015; Razmjou *et al*., 2018). Therefore, it is necessary to study the changes of these enzymes in IPM, because proper nutrition and adequate energy intake from food resources ultimately affect egg production and other biological activities. Due to the importance of digestive enzymes and nutrition as the important features of natural enemies, the present research was conducted on the sublethal effects of selected insecticides from different chemical groups on the amylolytic and proteolytic activity of *H. hebetor*. The main idea for this research was to determine safe and sustainable insecticides on this important biocontrol agent for their effective integration in IPM designs.

Materials and Methods

Insecticides

Five commercial formulations of fenvalerate, propargite, buprofezin, dayabon and palizin insecticides were used in the enzymatic study on *H. hebetor*. Information about the selected pesticides is given in Table I.

Table I. Insecticides selected for enzymatic studies on *H. hebetor*.

Insecticide	Trade name	Trade formulation	Manufacturer	Country
Fenvalerate	Sumicidin	EC 20%	Arasanj Shimi	Iran
Propargite	Omite	EC 54%	Aria Shimi	Iran
Buprofezin	Applaud	SC 40%	Nihon Nohyaku	Japan
Dayabon	-	EC 40%	Nano Fan-Avaran Daya	Iran
Palizin	-	SL 60%	Kimia Sabz-Avar	Iran

EC – emulsion concentrate, SL – soluble liquid, SC – suspension concentrate.

Rearing of *Ephestia kuehniella*

Flour moth eggs were from a private insectarium (Jalilian) from Eslamabad-e Gharb city in Kermanshah province, Iran, during 2018. Then, 0.25 g of the eggs were distributed on the surface of a wheat flour + bran mixture (750 g to 250 g) under the laboratory conditions at 20±1°C, 50±5% relative humidity (RH), and a relative dark (Abedi *et al*., 2012, 2014; Mahdavi and Saber, 2013). Under these conditions, the pest moth (Fig. 1B) showed five larval stages (Fig. 1C) with a life-cycle period of about 40 to 45 days.

Figure 1. A – female wasp of *H. hebetor*; B – female moth of *E. kuehniella*; C – larval stage of *E. kuehniella*.

Rearing of *Habrobracon hebetor*

Females of the parasitoid wasp (Fig. 1A) were obtained from a private insectarium (Jalilian) in Eslamabad-e Gharb city (Kermanshah province in Iran), during 2018. The wasps were quickly moved to a growth chamber in the laboratory and reared on *E. kuehniella* larvae at 20±1°C, 50±5% RH, and a photoperiod of 16:8 (L: D) h. The parasitoid was fed with a 20% honey solution (Rafiee-Dastjerdi *et al*., 2008; Abedi *et al*., 2012, 2014).

Enzymatic mixture preparation

Females of *H. hebetor* were first treated with a sublethal concentration (LC_{30}) of the selected insecticides for 24 h. Then, live females were carefully dissected in distilled water using a stereomicroscope, and the isolated midguts were placed in 1.5 ml microtubules containing 1% NaCl and homogenized in ice using a homogenizer; finally, the homogeneous mixture was centrifuged at 4°C at 15,000 rpm for 15 min. The obtained supernatant was stored at -20°C as an enzyme source (Saadat *et al*., 2014).

Protein assay

One of the main parts in the formula for determining the specific activity of the digestive enzymes (U) is the protein concentration (C), which was measured by the Bradford method (1976). According to this, the rate of absorption at 595 nm indicates the protein concentration in each sample. In this study, 600 µl of Bradford reagent was added to 20 µl of enzyme mixture in 1.5-ml microtubes, and three replications were performed. Finally, the protein content was determined by using the standard protein equation and comparing the absorption of the studied samples with it.

Alpha-amylase activity

Each unit contained 20 µl of enzyme mixture, 50 µl of substrate (starch 1%) and 250 µl of phosphate buffer, which were mixed together and maintained at room temperature for 30 min. After that, 50 µl of 3,5-dinitrosalicylic acid (DNSA) was added as an enzyme inhibitor and placed in a water bath at 90°C for 10 min. After 5 min of centrifugation at 16,000 rpm at 4°C, absorption was measured at 540 nm using a spectrophotometer. In addition, a blank sample was used for the control. All assays were performed in triplicate (Bernfeld, 1955).

General protease activity

As a substrate, 1% azocasein was used for measuring protease activity in the midgut of *H. hebetor* (Elpidina *et al*., 2001). Fifty µl of enzyme mixture and 80 µl of substrate were placed in an appropriate volume of phosphate buffer at room temperature for 60 min. Then, 100 µl of 30% trichloroacetic acid (TCA) was added as enzyme inhibitor and undigested azocasein was removed. The mixture was centrifuged in 4°C at 15000 rpm for 15 min. Finally, 100 µl of NaoH 2M was added to 100 µl of the prepared mixture and its absorbance was measured at 405 nm using a spectrophotometer. All treatments were performed in triplicate (Saadat *et al*., 2014).

Statistical analysis

Data were examined by one-way ANOVA and their means were compared by Tukey's test ($p<0.05$) with SPSS ver. 16 software (Meyer *et al*., 1986; Maia *et al*., 2000).

Results

The midgut status in *H. hebetor*

Based on a comparison of the digestive tract in *H. hebetor* with the other species in the family Braconidae, the midgut (proventriculus) is located at end of the crop, which is very narrow and connected to the cardiac valve (Fig. 2A). The midgut (Fig. 2B) is the main source of the digestive enzymes in *H. hebetor* and is connected at end to the Malpighian tubes (Fig. 2C), which are very small, and numbered ten. Finally, after

the Malpighian tubes, there is a third part of the digestive tract that includes the ileum, colon and rectum, ending at the anus.

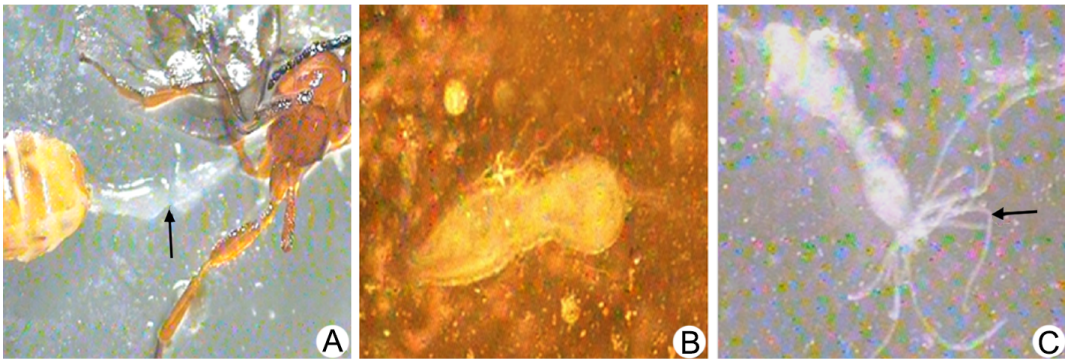

Figure 2. A – cardiac valve of *H. hebetor*; B – midgut of *H. hebetor*; C – Malpighian tubes of *H. hebetor*.

Protein concentration in the midgut

Protein concentration in the midgut of *H. hebetor* is shown in Table II. Based on analysis of variance (ANOVA), there was no significant difference among the control and all insecticides treatments ($F_{5, 12}$= 0.670); the highest amount of protein was in the control and was 0.6000±0.0103 mg/ml and the lowest was in the fenvalerate treatment (0.4147±0.0659 mg/ml). The decreasing trend was: control, palizin, dayabon, propargite, buprofezin, and fenvalerate.

Table II. Effect of insecticides on the protein concentration in the midgut of *H. hebetor.*

Treatment	Protein (mg/ml)
Control	0.6000±0.0103 [a]
Fenvalerate	0.4147±0.0659 [a]
Propargite	0.5141±0.0634 [a]
Buprofezin	0.5049±0.0250 [a]
Dayabon	0.5311±0.1684 [a]
Palizin	0.5774±0.0132 [a]

[a] – non-significant difference (Tukey's test, $p<0.05$)

Amylolytic activity in the digestive tract

The results of amylolytic activity in the midgut of *H. hebetor* are given in Table III. Based on ANOVA, there were significant differences among the different treatments and the control ($F_{5, 12}$= 14.695). The highest activity was observed in the control (0.0435±0.0023 mU/mg) and the lowest in the fenvalerate treatment (0.0277±0.0010 mU/mg). The differences between the control and dayabon and between propargite and palizin were not significant. The decreasing trend of the treatments was as follows: control, dayabon, palizin, propargite, buprofezin and fenvalerate.

Table III. Effect of insecticides on the amylolytic activity in the midgut of *H. hebetor.*

Treatment	Amylolytic activity (mU/mg)
Control	0.0435±0.0023 [a]
Fenvalerate	0.0277±0.0010 [c]
Propargite	0.0366±0.0019 [ab]
Buprofezin	0.0317±0.0009 [bc]
Dayabon	0.0426±0.0015 [a]
Palizin	0.0375±0.0015 [ab]

Different letters above the values show significant differences (Tukey's test, $p<0.05$)

Proteolytic activity in the digestive tract

The results of proteolytic activity in the midgut of *H. hebetor* are shown in Table IV. They indicated significant differences among the control and insecticide treatments based on ANOVA ($F_{5, 12}$= 99.278). The highest and lowest activities were observed in the control and propargite treatment (4.9817±0.0268 U/mg and 3.2231±0.0917 U/mg, respectively). Also, the differences between the control and palizin and between propargite and buprofezin were not significant. The decreasing trend of the treatments was: control, palizin, dayabon, fenvalerate, buprofezin and propargite.

Table IV. Effect of insecticides on the proteolytic activity in the midgut of *H. hebetor.*

Treatment	Proteolytic activity (U/mg)
Control	4.9817±0.0268 [a]
Fenvalerate	3.8645±0.0797 [c]
Propargite	3.2231±0.0917 [d]
Buprofezin	3.2605±0.393 [d]
Dayabon	4.3476±0.1159 [b]
Palizin	4.7541±0.0572 [a]

Different letters above the values show significant differences (Tukey's test, $p<0.05$)

Discussion

Different insecticides have contact, fumigant, stomach and repellent effects. Therefore, they play very important roles in the control of plant pests (Rafiee-Dastjerdi *et al*., 2013; Parsia Aref & Valizadegan, 2015; Razmjou *et al*., 2018). It is important to note that the compounds that have more effective insecticidal compounds have more negative effects on insects, including natural enemies and parasitoid wasps. Our research showed that dayabon and palizin as botanical insecticides showed low acute toxicity on the ectoparasitoid wasp *H. hebetor* compared to fenvalerate, propargite and buprofezin. This indicated that natural or botanical compounds are safer for natural enemies, which must be seriously considered in biological control and IPM programs.

Vinson (1969) examined the digestive tract of *Cardiochiles nigriceps* Viereck (Hym.: Braconidae; Cardiochilinae) and described a long esophagus with a thin-walled crop at its end. After these parts, the proventriculus was placed in front of the midgut. The Malpighian tubes were not obvious in the abdominal

cavity and appeared to be short. The results of his study were consistent with the results of our study on *H. hebetor*. Different species in the family Braconidae have phylogenetic relationships, which are also evident in the structure of various internal organs. Benham (1972) examined the digestive tract and reproductive system of the parasitoid *Eriborus molestae* Uchida (Hym.: Ichneumonidae) and found that the first part of the digestive tract consisted of a shallow mouth cavity with an elongated esophagus that entered the abdomen in the petiole area. There was a flexible and thin-walled crop that extended to the front of the stomach. In his study, the midgut was simple and surrounded by a series of distinct cylindrical muscles. Furthermore, the hindgut had three distinct parts, including the ileum, colon and rectum with two large and distinct areas and 24 to 32 Malpighian tubes in the abdominal cavity. Since the different species of the family Ichneumonidae such as *E. molestae* are phylogenetically related to Braconidae, there are some similarities in their digestive tracts.

To date, there are limited studies on the activity of digestive enzymes in *H. hebetor*. Saadat *et al*. (2014) studied the development of *H. hebetor* on the larvae of *Plodia interpunctella*, *Spectrobates ceratoniae* Zeller, *Helicoverpa armigera* (Hübner), *Ephestia kuehniella* and *Malacosoma disstria* L. in relation to enzyme activities. They found that keeping the wasps on stored pests was more effective due to the high hatch rate, suitable life cycle, high offspring sex ratio and weight dry mass. Their results showed that the best growth conditions for the wasps included meals rich in sugar and glycogen but that the best conditions for wasps grown on field pests were meals rich in terpenes and tannins. In another study, Borzoui *et al*. (2016) examined the survival and adaptation of *H. hebetor* on *E. kuehniella* and *H. armigera* larvae. They found that parasitism of the wasp was related to its enzymatic activities. In their study, the desired result was obtained when the wasp was kept on the fourth instar larvae of *H. armigeral*, because the α-amylase and protease enzymes of the parasitoid wasps showed high activity. Thus, they determined the highest percentage of parasitism on fourth instar *H. armigera* larvae.

The feeding characteristics of parasitoid wasps and their hosts affect digestive enzyme activity. In the present study, the activity of enzymes, including α-amylase and protease in the control, was at a good level, which confirms the results of above-mentioned studies. In general, it can be said that the higher activity of these enzymes in natural enemies, especially parasitoids, caused higher nutritional potential, which ultimately increases egg production and parasitism activity on their hosts' larvae. This could provide a new approach to discussing the effects of physiological characteristics in natural enemies and their relationship to their optimal performance in the control of plant pests. Unfortunately, there is no study about the direct effects of pesticides on the changes in the digestive enzymes of *H. hebetor*. Therefore, this research is a new project in this field, which could be continued on other natural enemies. The results of this study showed that the insecticides that had more acute and chronic toxicity for this parasitoid wasp reduced its digestive enzyme activity and, conversely, lower toxic treatments, due to their less destructive effects, affected the enzyme activity less than the control.

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СУБЛЕТАЛНИ ЕФЕКАТ ПЕТ КОМЕРЦИЈАЛНИХ ИНСЕКТИЦИДА НА АМИЛОЛИТИЧКУ И ПРОТЕОЛИТИЧКУ АКТИВНОСТ ВРСТЕ *HABROBRACON HEBETOR* SAY (HYMENOPTERA: BRACONIDAE)

МОХАМАД АСАДИ, ХУШАНГ РАФИ-ДАСТЏЕРДИ, ГАДИР НУРИ-ГАНБАЛАНИ, ХАСАНАЛИ ВАХЕДИ, БАХРАМ НАСЕРИ И МАХДИ ХАСАНПУР

Извод

Habrobracon hebetor Say је честа врста која се користи у биолошкој борби и има широк спектар домаћина у реду Lepidoptera, посебно из породица Noctuidae и Pyralidae. Исхрана је веома важан елемент у ефикасности природних непријатеља у интегрисаном управљању штеточинама (ИПМ). У приказаном истраживању, у лабораторијским условима проучавани су сублетални ефекти пет одабраних хемијских и ботаничких инсектицида, фенвалерата, пропаргита, бупрофезина, дајабона и пализина, на активност ензима амилазе и протеазе у средњем цреву ове важне ектопаразитоидне осе. Промене активности ензима испитиване су третманом женки оса LC_{30}, које су износиле 0,14, 7,01, 3,04, 9,58 и 2,53 mg a.i./mL, респективно. Добијени резултати су показали да ова паразитоидна оса има дугачко средње црево у свом дигестивном тракту. Такође, постојале су значајне разлике међу ензимским третманима ($F_{5,12}$ =14,695 и 99,278) осим концентрације протеина ($F_{5,12}$ = 0,670). Што се тиче ензима амилазе, највеће и најниже активности су добијене у контролном и фенвалератном третману (0,0435±0,0023 mU/mg према 0,0277±0,0010 mU/mg). Поред тога, највећа протеолитичка активност је забележена у контроли (4,9817±0,0268 U/mg), а најнижа у третману пропаргитом (3,2231±0,0917 U/mg). Пажљивом анализом резултата, даиабон и пализин су показали најниже негативне ефекте на ензиме и могли су се применити заједно са овом паразитоидном осом у ИПМ дизајну.

Received: October 13th, 2022
Accepted: November 30th, 2022