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Research Article

Changes of phenoloxidase activity in hemocytes of *Helicoverpa armigera* (Lepidoptera: Noctuidae) exposed to sub-lethal concentrations of insecticides

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Abstract: The cotton bollworm, *Helicoverpa armigera* (Hübner), is one of the most important pests of crops. Like other insects, this insect has an immune system against external threats such as various diseases, parasitoids, and chemical compounds. Phenoloxidase is a key factor in the immunity of insects and other arthropods. The present study investigated the lethal effects of four insecticides, indoxacarb, profenofos, chlorfluazuron, and hexaflumuron, and their effects on phenolic oxidase activity in cotton bollworm. Among the studied insecticides, in two bioassays (mixture of insecticide with artificial food and immersion of sugar beet leaves), Chlorfluazuron had the highest toxicity (LC_{50} = 1.71 and 3.11 mg ai/liter, respectively). Also, the highest phenol-oxidase activity at 24 h was for larvae treated with chlorfluazuron and hexaflumuron. Also, when treated with chlorfluazuron and hexaflumuron, the larvae fed on an artificial diet had higher phenoloxidase activity than those fed on sugar beet cultivars.

Keywords: cotton bollworm, immune system, Phenoloxidase enzyme, sugar beet cultivars

Introduction

One of the most important issues related to agriculture and food production is the protection of crops against agricultural pests. If these pests are not controlled or mismanaged, quantitative and qualitative reduction of agricultural output will be inevitable. Cotton bollworm is an important oligophagous pest of crops in many parts of the world (Fitt, 1989). Widespread oligophagous, high mobility, facultative diapause, high reproduction, and resistance potential against a wide range of insecticides have made this insect one of the most important agricultural pests (Fitt, 1989; El-Wakell, 2003). Finding new, effective, and environmental protection standard methods for managing this pest, as well as other agricultural pests, seems necessary. Insects have a highly efficient immune system that depends on the species, age, food quality, and reproductive characteristics of pests (Adamo *et al*., 2001). When an external agent enters an insect hemocele, the immune system reacts to invasive organisms with immune responses (Gillespie *et al*., 2000; Lavine and Strand, 2002). The phenoloxidase enzyme is one of the key enzymes in the immune system of many arthropods that is activated soon after

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introducing foreign agents or injuries to the insect body (Gillespie *et al*., 1997). In order to know the biochemical properties of phenoloxidase enzyme, optimal activity conditions, inhibition of this enzyme activity by disrupting its normal process, and ultimately effective control of agricultural pests, several studies on the effect of various insecticides on insect immune system and especially the activity of this enzyme have been conducted. Zibaee *et al*. (2009) reported that under the influence of temperature, the number of hemocytes, nodule formation, and phenoloxidase enzyme activity of the Sun bug, *Eurygaster integriceps* (Puton) (Hemiptera: Scutelleridae) were affected after inoculation by *Beauveria bassiana* (Bals-Criv) (Hypocreales: Cordycipitacea). Rahimi *et al*. (2013) studied the interaction of two insect growth regulators (pyriproxyfen and hexaflumuron) with *B. bassiana* on the immune responses of Mediterranean flour moth, *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae). Although phenoloxidase activity was not significantly different in the larvae treated with pyriproxyfen compared to the control, a significant difference in enzyme activity was reported between one and three hours after treatment by hexaflumuron. Mirhaghparast *et al*. (2015), in the study of immune responses of Rice stem borer, *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) treated with the hexaflumuron, observed that along with the increase in the number of hemocytes, the activity of phenoloxidase enzyme was increased at different interval times as well as at all concentrations. The aim of this research is 1) Investigation of the phenoloxidase enzyme activity in cotton bollworm as an important factor in the immune system of arthropods, 2) Identification of the effective insecticidal compounds against cotton bollworm by investigating the activity of this enzyme in treated larvae, as well as under different thermal conditions, 3) Introduction of the insecticidal compounds that would be recognized by insect immune systems and cause increasing or decreasing of this enzyme activity and 4) Recognizing and using inhibitors of this enzyme along with the insecticides and subsequent targeting the immune system and inhibiting of this enzyme in order to manage this pest.

Materials and Methods

Insect breeding

To obtain a sufficient population of the target pest, a colony of cotton bollworm supplied by the Department of Plant Protection, University of Tabriz, Iran, was reared on an artificial diet for three generations at optimal conditions (26 ± 2 ºC, relative humidity 60 ± 10 % and 16:8 h photoperiod)

Insecticides

The insecticides used in the experiments were: Indoxacarb (15% EC) (Avaunt®) produced by G-CERTZ Company, India; Profenofos (50% EC) (Curacron®) and Hexaflumuron (10% EC) (Consult ®) made by Golsam Company, Gorgan, Iran; Chlorfluazuron (5% EC) (Atabron ®) produced by ISK Company, Japan.

Ingestive toxicity bioassays

The lethal effects of each insecticide were investigated on the 4th instar larvae of cotton bollworm. The larval instars were differentiated based on the head capsule size. The experiments were conducted using artificial food mixed with insecticides and immersing leaf pieces of sugar beet cultivars in a toxic solution. After dose-setting tests, the insecticides were prepared in five main concentrations. Distilled water was used as the control treatment. In the first method, each insecticide solution was mixed with artificial food in a ratio of 1: 9 (toxic solution: food). In the second method, the pieces of sugar beet leaves were immersed in the toxic solution for 10 seconds. After drying, leaf pieces were given to the larvae for feeding. Twenty 4th instar larvae were selected randomly for each concentration and control treatment. For each insecticide, 360 larvae (4th instar) were used in each experimental method. The lethality evaluation experiments of each insecticide were repeated three times, and larval mortality was recorded at 24-hour intervals for the indoxacarb, 48 hours for the profenofos, and 72 hours for hexaflumuron and chlorfluazuron.

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Phenoloxidase extraction and activity assay

To extract the hemolymph, the fleshy third pair foot of 4th instar larvae anesthetized on ice was gently cut with a scalpel. According to Azambuja *et al*. (1991), the extracted hemolymph was added into a 2 ml Eppendorf tube containing 200 µl of anticoagulant solution (0.01 M Ethylene thiamine tetra-acetic acid, 0.1 M Glucose, 0.062 M NaCl, 0.026 M Citric acid). The collected sample was centrifuged at 12000 rpm for four minutes at 4 °C, and the upper phase was removed. Subsequently, 100 µl cold phosphate buffer solution was added and centrifuged at 15000 rpm for 4 minutes at 4 °C. The above phase was collected and incubated for 30 min at 30 °C, then 20 µl of 10 mM L-dopa substrate was added to it and incubated for 5 minutes at 30 °C. Finally, absorbance was recorded at 490 nm using a microplate reader in three replications (Zibaee *et al*., 2011). Forty 4th instar larvae were examined for each replication.

Impact of insecticides on phenoloxidase activity

The effects of sub-lethal concentrations of studied insecticides on phenoloxidase activity were evaluated. The experiments were conducted using an artificial diet mixture with insecticides and dipping the sugar beet cultivar leaf disks. Sixty 4th instar larvae were selected and treated with the LC_{30} value of each insecticide. After 24 hours of treatment, phenoloxidase activity was measured in survived larvae in three replications.

Interaction of insecticides with diets

To investigate the effect of different diets on the activity of phenoloxidase enzyme in larvae treated by insecticides, dipping the sugar beet cultivar leaves disks in a toxic solution. Seeds of three sugar beet cultivars, Sandrina, Dorta, and Pars (native cultivars), were supplied by the Seed and Plant Improvement Institute, Karaj, Iran. The seeds of each cultivar were immersed in distilled water for 24 h, then cultivated in the greenhouse of the Department of Plant Protection, University of Tabriz, under controlled conditions. In the 4-6 leaf stage, the healthy and fresh leaves of each cultivar were cut and located in separate containers. One first instar cotton bollworm larva (Neonate larvae) was transferred to each container. In 4th instar larval age, they were treated with LC_{30} of each insecticide, and the activity of the phenoloxidase enzyme was evaluated according to the method mentioned above.

Estimation of total protein

Total protein concentration in samples was measured using Bradford's (1976) method and bovine serum albumin as the standard protein.

Statistical analysis

Data were analyzed using SPSS software. The means were compared by Duncan's multiple range test (5%). Also, lethal concentration values for insecticides were analyzed using SPSS software (Probit analysis). Numerical calculations and regression relations were performed using Excel software to draw the graphs. All experiments were performed in three replications.

Results

Ingestive toxicity bioassays

The effect of indoxacarb, profenofos, chlorfluazuron, and hexaflumuron by two methods (mixed with artificial diet and treatment of sugar beet leaves of cultivars, Sandrina, Dorta, and Pars, in insecticide solution) on 4th larvae of cotton bollworm is (shown in Tables 1 and 2, respectively). According to the probit analysis, the confidence range of LC_{50} values of the treatments did not overlap, so it can be concluded that there is a significant difference among the treatments (Fig. 1). The chlorfluazuron had higher toxicity on cotton larvae in both bioassays, with the lowest value of LC_{50} (1.71 mg/l) compared to the other three insecticides. Also, indoxacarb and hexaflumuron were ranked second and third, respectively. The profenofos with the highest value of LC_{50} (905.74 mg/l) had the lowest toxicity against cotton bollworm larvae. According to the results, the lethal values of all tested insecticides were much lower in

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bioassays by insecticides mixed with artificial food compared to bioassays by treating the host plant leaves. In other words, the plant leaves required higher concentrations of insecticides in the bioassay (Fig. 1).

Impact of insecticides on the phenoloxidase activity

The variance analysis showed that there was a difference between treated and non-treated larvae enzyme activity ($F_{4,14}$ = 545.222, P < 0.0001) (Table 3). As shown in Fig. 2, the activity of phenoloxidase enzyme in larvae treated with chlorfluazuron, hexaflumuron, and profenofos increased in comparison with controls, and there was a significant difference between the effects of these three insecticides. But no significant difference was observed between indoxacarb and non-treated larvae. The results showed that chlorfluazuron and hexaflumuron from the IGR group, due to their nature and mode of action, 24 hours after treatment, significantly increased the activity of the phenoloxidase. On the other hand, although the insecticide profenofos caused a significantly increased phenoloxidase activity, this increase was lower than that of chlorfluazuron and hexaflumuron.

Interaction of insecticides and diets on the phenoloxidase activity

The results of interactions of two insecticides, chlorfluazuron, and hexaflumuron, in four different diets, including an artificial diet and three sugar beet cultivars (Pars, Dorta, and Sandrina) on phenoloxidase activity, showed that there were significant differences among the treatments ($F_{7.24}$ = 651.054, P < 0.0001) (Table 4). Also, there was a significant difference between diets and insecticides. According to Fig. 3, there was a significant difference between the four diets in the chlorfluazuron treatment. On the other hand, phenoloxidase enzyme activity in larvae raised on artificial food is much higher than in larvae fed on sugar beet cultivars. Although there is a significant difference between sugar beet cultivars regarding enzyme activity, this difference is small compared to artificial food. In the treatment with hexaflumuron, larvae fed on artificial food showed higher enzyme activity levels and a significant difference in the probability level of 5% compared to larvae fed on sugar beet cultivars. However, there was no significant difference between sugar beet cultivars regarding enzyme activity as opposed to the hexaflumuron treatment.

Table 1 Ingestive toxicity of insecticides on 4th instar larvae of *Helicoverpa armigera*.

Insecticide	N[1]	χ^2	Slope ± SE	LC_{30} (mg/l) (95% FL)[2]	LC_{50} (mg/l) (95% FL)	LC_{90} (mg/l) (95% FL)
Indoxacarb	360	0.47	3.6 ± 0.34	3.84 (3.25-4.33)	5.39 (4.83-5.97)	12.39 (10.47-15.93)
Profenophos	360	0.39	5.8 ± 0.38	737.4(675.0-787.6)	905.7(852.5-966.2)	1497(1329.5-1806)
Chlorfluazuron	360	0.08	1.4 ± 0.26	0.79 (0.54-1.04)	1.71 (1.34-2.15)	11.18(7.57-20.32)
Hexaflumuron	360	0.40	1.9 ± 0.07	4.67(3.32-5.86)	8.50 (6.95-10.02)	36.75(27.03-60.11)

[1.] Number of used insects in the bioassay.
[2.] Fiducial Limits.

Table 2 Toxicity of the tested insecticides on *Helicoverpa armigera* in leaf disc contact bioassay.

Insecticide	N[1]	χ^2	Slope ± SE	LC_{30} (mg/l) (95% FL)[2]	LC_{50} (mg/l) (95% FL)	LC_{90} (mg/l) (95% FL)
Indoxacarb	360	0.79	3.45 ± 0.46	5.97 (5.19-6.62)	8.13 (7.39-8.92)	17.27 (14.68-22.07)
Profenophos	360	0.27	6.87 ± 0.77	917.02 (841.5-977.3)	1121.2 (1057.2-1195.4)	1832.51 (1626.29-2220.70)
Chlorfluazuron	360	0.30	1.65 ± 0.09	1.49 (1.03-1.9)	3.11 (2.47-3.85)	18.57 (12.56-34.50)
Hexaflumuron	360	0.40	2.29 ± 0.10	6.98 (5.2-8.5)	11.83 (9.93-13.82)	42.98 (32.71-66.65)

[1.] Number of used insects in the bioassay.
[2.] Fiducial Limits.

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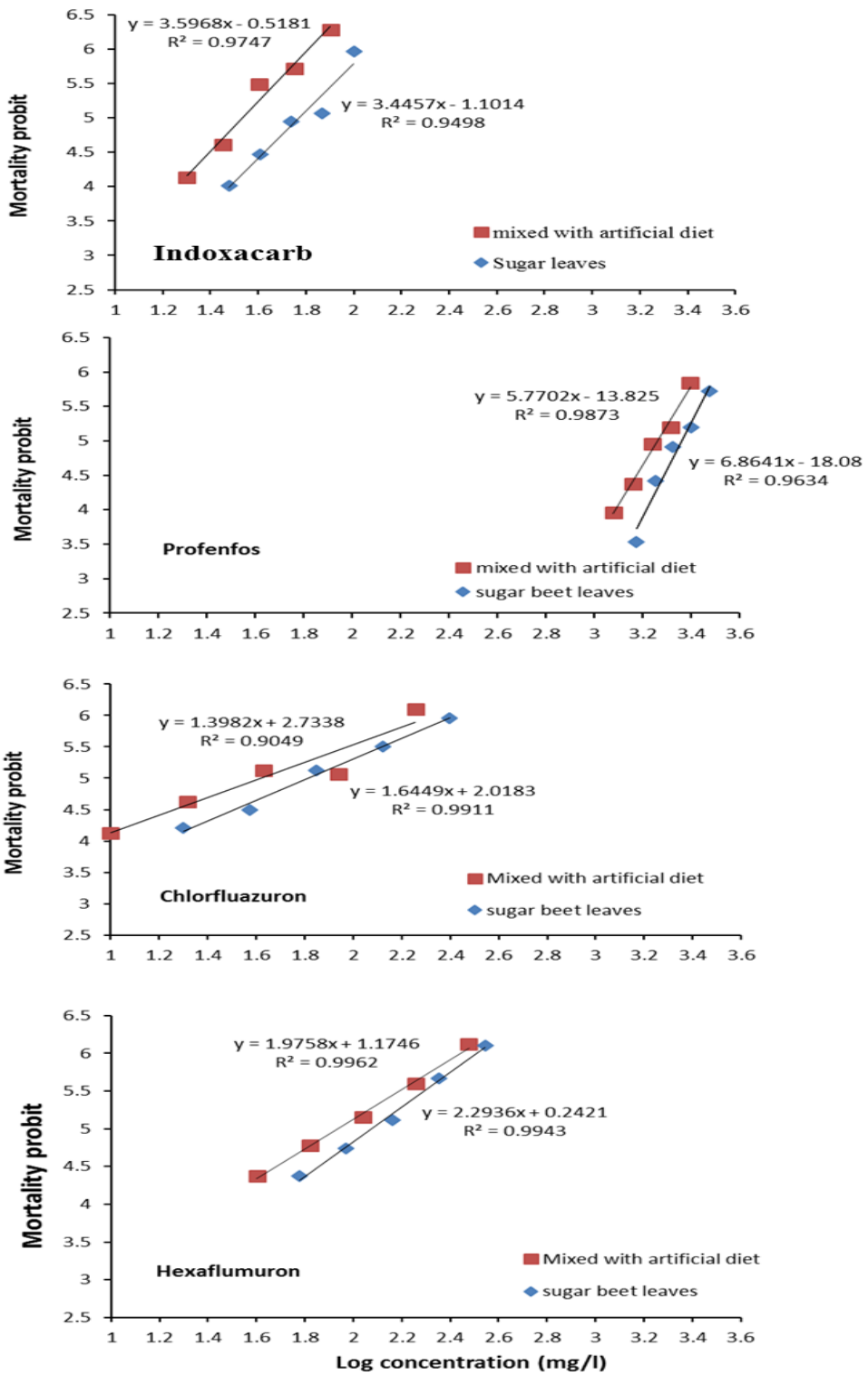

Figure 1 Dose-response relationship of indoxacarb, profenofos, chlorfluazuron and hexaflumuron in two bioassay tests (ingestive and contact methods) on the 4th instar larvae of *Helicoverpa armigera*.

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Table 3 Analysis of variance of four insecticides on phenoloxidase activity in cotton bollworm larvae compared with control.

source	df	Sum of square	Mean square	F	P-value
Treatment	4	0.060	0.015	545.222	< 0.0001
Error	10	0.000	0.000		
Total	14	0.060			

CV% = 0.147.

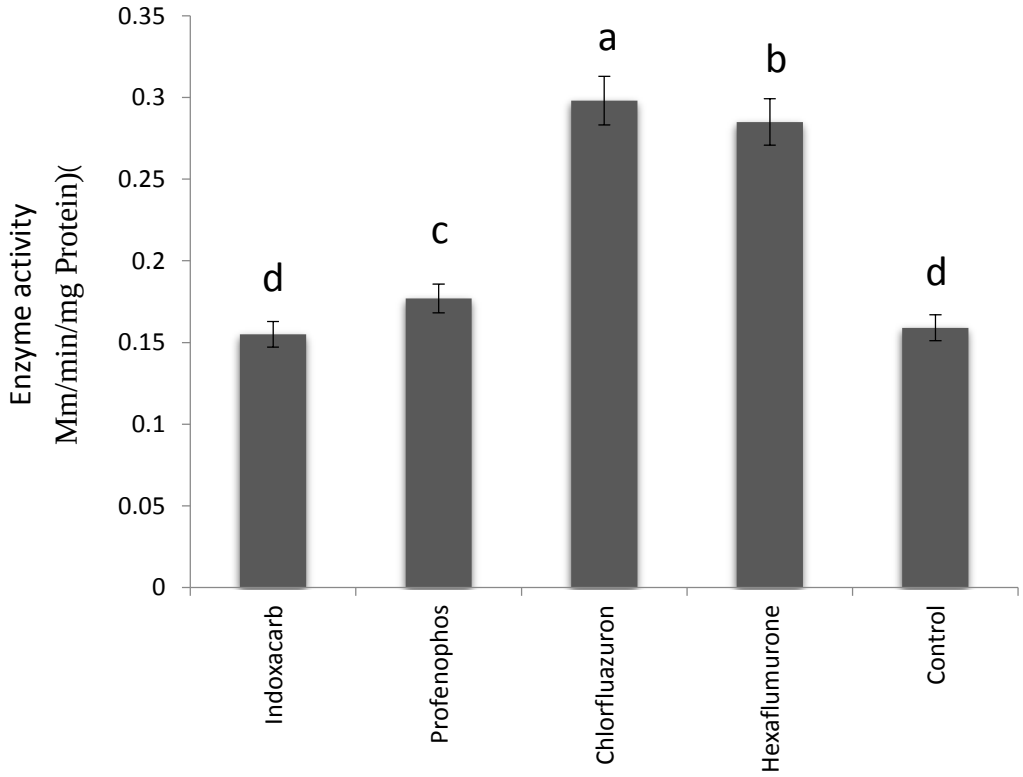

Figure 2 Phenoloxidase activity of the 4th instar larvae of *Helicoverpa armigera* by the ingested insecticides compared to non- treated larvae.

Table 4 Analysis of variance of chlorfluazuron and hexaflumuron and four artificial diets on phenoloxidase activity 4th instar larvae of *Helicoverpa armigera*.

Source of variations	df	Sum of square	Mean of square	F	P-value
Cultivar	3	0.045	0.015	651.054	< 0.0001
Insecticide	1	0.001	0.001	31.930	< 0.0001
Cultivar×Insecticide	3	0.000	0.000	4.984	< 0.0001
Error	16	0.000	2.308		0.013
Total	24	1.163			

CV%= 0.123.

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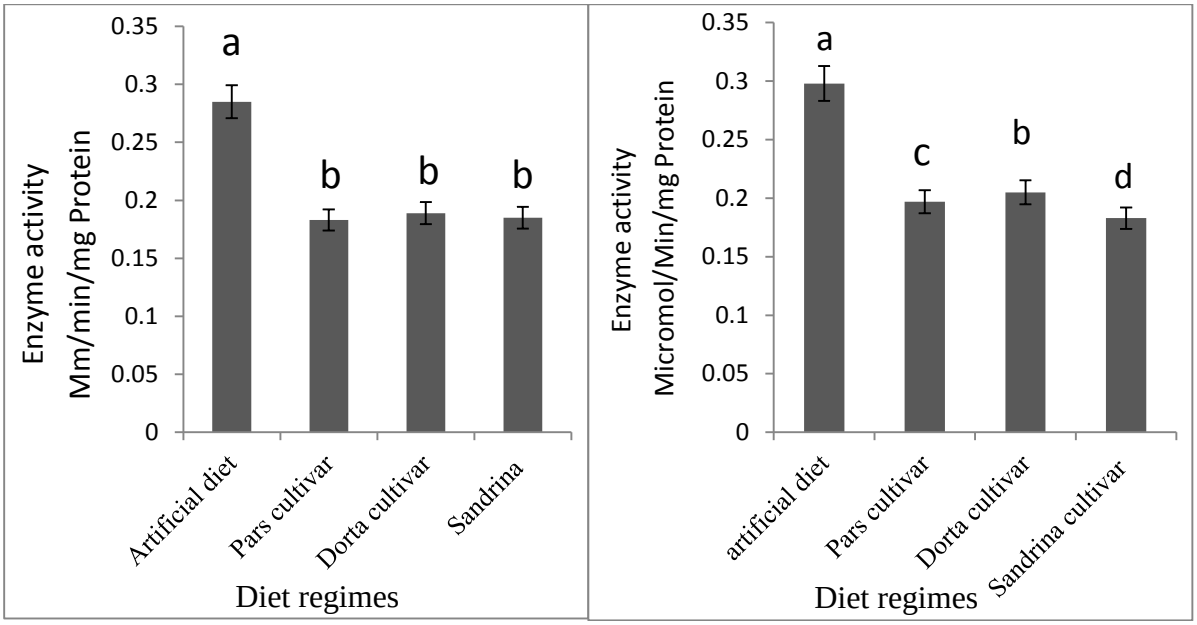

Figure 3 Impact of chlorfluazuron (right) and hexaflumuron (left) on phenoloxidase activity of the 4th instar larvae of *Helicoverpa armigera* fed by diet regimes.

Discussion

Ingestive toxicity

Several studies have been conducted on the lethal effects of insecticides on cotton bollworm. Rafiei Dastjerdi *et al*. (2008) tested hexaflumuron and profenofos insecticides on first-instar larvae by combining the insecticide with an artificial diet and soaking the host leaf in an insecticide solution. In their studies, the values of LC_{50} for profenofos in combining the chemical with artificial food method and the leaf soaking were 3.69 and 9.55 mg/l, respectively, and LC_{50} of hexaflumuron was 0.31 and 0.46 mg/l, respectively. In this study, in the method of mixing an insecticide with artificial food, the LC_{50} values for hexaflumuron and profenofos insecticides were 8.50 mg/l and 905.74 mg/l, respectively. But, in the larval feeding method from sugar beet leaves treated with insecticide, the LC_{50} values were recorded as 11.83 mg/l for hexaflumuron and 1121.21 mg/l for profenofos. This difference can be related to the difference in the studied larval instar because the younger larvae can be much more susceptible to insecticides than older larvae. However, Taleh *et al*. (2015) examined the toxicity of hexaflumuron on the 4th instar larvae of cotton bollworm by mixing the insecticide with artificial food and reported the LC_{50} and LC_{90} values 8.47 mg/l and 82.26 mg/l, respectively, which are very close to the values obtained in the present study. The concordance of results can be related to the similarity of used insecticide and pest species. Bakr *et al*. (2010) investigated the lethal and non-lethal effects of the chlorfluazuron insecticide on the Red flour beetle, *Tribolium castaneum* (Herbts) (Coleoptera: Tenebrionidae) and reported the LC_{50} = 1.2 ppm for this pest, which is very close to the results of the present study. Moadeli *et al*. (2014) assayed the lethal and non-lethal effects of indoxacarb insecticide on larvae of Beet armyworm, *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) by soaking the leaves of the host plant and LC_{50} and LC_{90} values were recorded 2.51 mg/l and 38.828 mg/l respectively, which are different from the results of the current study. These differences can be attributed to the differences in the target pest species and the testing method.

Impact of insecticides on the phenoloxidase activity

Mirhaghparast *et al*. (2015), in the study of immune responses of *C. suppressalis* to

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treatment by hexaflumuron, observed that the activity of this enzyme increased at different interval times and in all concentrations. Assar *et al*. (2016), examining the biochemical effects of the insecticides teflobenzuron and hexaflumuron on the 4th instar larvae of Sugar beet cutworm *Spodoptera littoralis* (Lepidoptera: Noctuidae), reported that 72 hours after treatment with both insecticides, the levels of phenolic oxidase and chitinase increased significantly. Taha and Al-Hadek (2017), studied the biochemical effects of two insecticides, chlorfluazuron, and diflubenzuron, on the 2nd instar larvae of Cutworms (*Agrotis* spp.) and reported that the activity of phenoloxidase in larvae treated by chlorfluazuron increased significantly after 24 h. According to the results of the present study and previous research, it can be concluded that IGRs, especially chitin synthesis inhibitors, due to the mode of action and uptake by hemolymph, can change the level of phenolic oxidase activity and subsequently stimulate the insect's immune system. Thus, identifying the function of the insect immune system in the presence of these insecticides can be very effective in controlling pests.

Interaction of insecticides and diets on the phenoloxidase activity

Limited studies have been performed on the interaction effects of different diets on phenolic oxidase activity. Abisgold and Simpson (1987) found that feeding of Asian locust, *Locusta migratoria* (L.) (Orthoptera: Acrididae) on a diet containing 28% protein increased absorbance of amino acids from the intestine immediately after feeding, subsequently increased the concentration of proteins in the hemolymph. Adamo *et al*. (2001) studied phenoloxidase activity in males and females of Texas field cricket, *Gryllus texensis* (Cade and Otte) (Orthoptera: Gryllidae) and reported that this enzyme activity decreases in males with age. However, in females, with increasing age and reaching the reproductive stage, the activity of this enzyme increases to its highest level. This finding may be related to increases in the feeding of female insects during reproduction. Srygley *et al*. (2009), in a study on the migratory population of the Mormon cricket *Anabrus simplex* Haldeman (Orthoptera: Tettigonidae), found that 45 minutes after feeding on food containing 42% protein, the concentration of hemolymph proteins increased gradually to 6 h after feeding, and followed with an increase in phenoloxidase activity, ultimately reached to its highest level. They concluded that access to high-protein foods increases total hemolymph protein concentration and phenolic oxidase levels. These researchers also reported that strong and high body mass could affect the speed and quality of movement and the function of the immune system.

The phenoloxidase is inactive in the hemolymph of many arthropods, including insects, and is activated when a wound or foreign invader enters the hemolymph. In other words, increasing the activity level of this enzyme is to inhibit the foreign agent. After removing the foreign agent, the activity of this enzyme returns to its original state. Many agricultural pests can be controlled by identifying the biochemical properties of this enzyme and finding ways to change its activity. According to the findings of this study and the results of other researchers, it can be said that the nature and quality of the diet can significantly affect the immune system of insects by increasing or decreasing the number of proteinic substances in the body. Therefore, identifying the appropriate plant cultivars that affect the immune system and especially the activity level of the phenoloxidase enzyme to control the pest seems helpful.

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تغییرات فعالیت فنل‌اکسیداز در هموسیت‌های کرم غوزه پنبه *Helicoverpa armigera* (Lepidoptera: Noctuidae) در معرض غلظت‌های زیرکشنده حشره‌کش‌ها

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چکیده: کرم غوزه پنبه *Helicoverpa armigera* (Hübner) یکی از آفات مهم محصولات کشاورزی محسوب می‌شود. این حشره مانند سایر حشرات در برابر تهدیدهای خارجی از قبیل انواع بیماری‌ها، پارازیتوئیدها و ترکیبات شیمیایی دارای سامانه ایمنی هستند. یکی از فاکتورهای کلیدی در ایمنی حشرات و هم‌چنین سایر بندپایان آنزیم فنل‌اکسیداز می‌باشد. در مطالعه حاضر اثرات کشندگی چهار حشره‌کش ایندوکساکارب، پروفنوفوس، کلرفلوآزورون و هگزافلومورون روی لارو سن چهارم کرم غوزه پنبه و هم‌چنین تأثیر آن‌ها بر میزان فعالیت آنزیم فنل‌اکسیداز بررسی شد. کلرفلوآزورون در دو روش زیست‌سنجی (مخلوط حشره‌کش و غذای مصنوعی و آغشته کردن برگ گیاه چغندرقند) با داشتن کم‌ترین میزان LC_{50} به‌ترتیب ۱/۷۱ و ۳/۱۱ میلی‌گرم ماده مؤثره بر لیتر بیش‌ترین سمّیت را نسبت به سایر حشره‌کش‌ها در برابر کرم غوزه پنبه داشت. در بررسی تأثیر حشره‌کش‌ها بر میزان فعالیت آنزیم نیز بیش‌ترین میزان فعالیت آن پس از ۲۴ ساعت در لاروهای تیمار شده با دو حشره‌کش کلرفلوآزورون و هگزافلومورون گزارش شد. هم‌چنین در این پژوهش تأثیر رژیم‌های غذایی مختلف بر فعالیت آنزیم فنل‌اکسیداز لاروهای تیمار شده بررسی شد و نتایج نشان داد که لاروهایی که روی غذای مصنوعی تغذیه کرده بودند در مقایسه با ارقام چغندرقند، در تیمار با هر دو حشره‌کش کلرفلوآزورون و هگزافلومورون بیش‌ترین میزان فعالیت آنزیم را داشتند.

واژگان کلیدی: کرم غوزه پنبه، سیستم ایمنی، آنزیم فنل‌اکسیداز، ارقام چغندرقند

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