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Research Article

Impact of pyriproxyfen and methoxyfenozide on hemocytes of the Mediterranean flour moth, *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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Abstract: The Mediterranean flour moth, *Ephestia kuehniella* Zeller, is one of the major pests in stored products worldwide. Several problems assossiated with the use of conventional insecticides have strongly demonstrated the need for applying alternative safe compounds such as insect growth regulators (IGRs). In the present study, growth regulating activity and hematological effects of pyriproxifen and methoxyfenozide were evaluated on *E. kuehniella* larvae. Effects of the insecticides were evaluated under laboratory conditions set at 26 ± 1 °C and 75% RH. Findings indicated that inhibition dose for fifty percent of population (ID_{50}) was equal to 0.16 µg/mg larvae for pyriproxifen and 0.4 µg/mg larvae for methoxyfenozide, showing the considerable growth regulating effect on two-day-old fifth instar larvae. Then, influence of estimated doses were investigated on the insect hemocytes including total hemocyte count (THC) and differential hemocyte count (DHC). THC and the proportion of plasmatocytes were decreased as pyriproxifen doses increased, while, the granulocytes level was increased. In contrast, application of sublethal doses of methoxyfenozide caused a conciderable increase in THC and the plasmatocytes density, while, number of granulocytes was decreased. Since the total number of hemocytes and the proportion of plasmatocytes are very crucial in immune responses of insects, pyriproxifen could be used as an immunosuppressive pesticide in integrated control of *E. kuehniella*.

Keywords: IGRS, Total hemocyte count, Differential hemocyte count, Plasmatocyte, Granulocyte

Introduction

Insect immune system is known to involve both cellular and humoral factors which together form a potent defense against invading organisms (Gillespie *et al*., 1997; Lavine and Strand, 2002). The cellular immune responses are functionally referred to as hemocyte-mediated processes including phagocytosis, nodule formation and encapsulation (Strand and Pech, 1995; Schmidt *et al*. 2001).

A series of vital inter connections exist among physiological systems in insects (Nation, 2002). Beckage (2008) has completely revised bidirectional connections between the immune system and the nervous system in insects. Also, Beckage (2008) and Adamo (2006) reviewed evidences showing that hormones, many of which are directly or indirectly regulated by the central nervous system (CNS) (Nijhout, 1998), influence immune functions.

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Ecdysteroids are well known for their crucial role in the regulation of many developmental and physiological processes (Gade *et al*., 1997) and are considered as potential specific target sites for pest control (Dinan, 1989). Besides their predominant role as molting hormones, ecdysteroids also mediate both cellular and humoral immunity in insects (Flatt*et al*., 2008; Franssens *et al*., 2006). Also, in addition to the role of juvenile hormone (JH) in molting and metamorphosis, this compound is involved in a variety of other biological activities like immune responses (Rantala *et al*., 2003). In spite of these facts, very little work has been carried out on the influence of insect hormones and their synthetic analogues on the hematology and thereby immunological properties of insects. Franssens *et al*. (2006) showed that in contrast to ecdysone stimulation, treating third instar larvae of the flesh fly *Neobellieria bullata* Parker with JH or the JH analogues, fenoxycarb and pyriproxyfen, significantly reduced their ability to form nodules in response to laminarin. Jalali Sendi and Salehi (2010) indicated that methoprene (ZR-515), a juvenile hormone analogue, considerably declined total number of hemocytes in *Papilio demoleus* L. Immunosuppressive action of pyriproxyfen on *Bt*-infected *Plutella xylostella* L. was also proved by Kwon and Kim (2007). Figueiredo *et al*. (2006) showed the antagonistic action between azadirachtin, an ecdysteroid antagonist, and 20-hydroxyecdysone (20E) in phagocytosis process of *Rhodnius prolixus* Stal. It was also shown that JH I caused a significant reduction in total number of hemocytes as well as major structural changes to important cell types, i.e. plasmatocytes and granulocytes in two lepidopteran *Hyphantria cunea* Drury and *Glyphodes pyloalis* Walker (Zibaee and Jalali Sendi, 2012). Therefore, these reports present the regulatory role of insect hormones or their synthetic analogues on insect immune components especially hemocytes. However, there is no report on the impact of insect growth regulators (IGRs) on the hemocytes of the Mediterranean flour moth, *Ephestia kuehniella* Zell.

E. kuehniella is considered as a serious cosmopolitan pest of stored products, particularly flour (Brindley, 1930). Because of environmental consideration, alternative approaches to neurotoxic insecticides, as well as safe, effective, and sound integrated pest management strategies are developed pest control agents such as IGRs (Hami *et al*., 2005). These compounds are biorational insecticides with novel modes of action which disrupt the physiology and development of the target pest and are also more selective and generally less toxic to non-target organisms than conventional insecticides (Biddinger and Hull, 1995; Gurr *et al*., 1999).

Pyriproxyfen, as an IGR, mimics the action of JH on a number of physiological processes, and is a potent inhibitor of embryogenesis, metamorphosis and adult formation (Ishaaya and Horowitz, 1992). This pesticide is widely used all over the world against many economically important agricultural pests (Koehler and Patterson, 1991; Ishaaya and Horowitz, 1992; Ghasemi *et al*., 2010). Methoxyfenozide, as an important member of the ecdysone agonist family, is highly specific to lepidopteran pests with low toxicity towards other insect orders (Smagghe *et al*., 2003).

The present work was designed to compare the growth regulating activity of pyriproxyfen and methoxyfenozide on two-day-old fifth instar larvae of *E. kuehniella*. Also, effects of estimated doses were assessed on total and differential hemocyte counts.

Materials and Methods

Insect culture

Eggs of *E. kuehniella* were provided by the Insectarium of Scientific and Industrial Research Organization of Iran. Each 300 one-day-old eggs were reared in a plastic container jar (25 × 15 × 10 cm) half filled with a mixture of equal amounts of wheat flour and bran. Powdered yeast (5 g) was also added to each jar. The rearing conditions were 26 ± 1 °C, 75% RH in darkness.

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Chemicals

Stock solution of pyriproxyfen (Admiral® 10%EC, Sumitomo chemical Co., LTD, Tokyo, Japan) was prepared in acetone (Merck, Germany) and then diluted to the desired concentrations for use in bioassays. Combination of distilled water and acetone (4:1 V/V) was used as solvent to obtain final doses of methoxyfenozide (Prodigy® 24%SC, Dow Agrosciences, Indiana, U.S.A).

Dose-response bioassays

To determine the toxicity values, two-day-old fifth instar larvae of *E. kuehniella* were topicaly treated with 1 µL of different concentrations of tested compounds. Before treatment, the larvae were placed on ice and immobilized. After a preliminary dose-setting trials, logarithmic series of dilutions were offered to identify the effective range for 5–95% mortality (Robertson *et al*. 2007).The final doses were applied on the second thoracic sternum of larvae using Hamilton syringe (Burkard Co., England). Control groups received 1 µL of the solvent alone. Ten larvae weighing 20 ± 1 mg were used in each cohort. All treatments and control insects were kept at 26 ± 1 °C and 70% RH in darkness. There were four replications per dose and the whole experiment was repeated twice. The inhibition doses (IDs) were estimated based on the percentage of adult emergence.

Total hemocyte count (THC)

To count total hemocytes of the larvae treated with estimated doses of pyriproxyfen (0, 0.05, 0.1 and 0.16 µg/mg larvae) and methoxyfenozide (0, 0.23, 0.32 and 0.4 µg/mg larvae), 2 µL aliquots of their hemolymph was mixed immediately in an eppendorf tube with 98 µL of Tyson solution (NaCl 2.72 mM; Na_2SO_4 8.96 mM; glycerol 43.68 mM; methyl violet 0.061 mM). Cell counting was conducted at intervals of 6, 12, 24 and 48 h post treatment using a standard Neubauer hemocytometer. The cells were counted using a light microscope (Olympus BX51) and number of total hemocytes was calculated.

Differential hemocyte count (DHC)

For DHC, drops of hemolymph were obtained from the proleg of the larvae 24 h after treatment with subjected IGRs and the fresh smears were prepared on a clean glass slide. The air dried smears were stained with Giemsa stain (diluted 7 times with distilled water and filtered before use) for 25 min, rinsed in saturated solution of lithium carbonate for 30 sec for red staining structures and subsequently washed in distilled water. After drying, permanent microscopic slides were prepared using Canada balsam. The hemocytes were observed under a light microscope (Olympus BX51) and differential countings of hemocyte morphotypes were performed by classifying 200 hemocytes per smear (Arnold and Hinks, 1976). Also, number of hemocytes undergoing mitotic division was recorded in each 200-cell of a smear.

Statistical analyses

The ID values and their 95% confidence limits were calculated from probit regressions using the POLO-PC software (LeOra Software). Data from hematology assays were analyzed using the SPSS program version 16.0 for analysis of variance (ANOVA) and the means were grouped using Tukey's test ($P < 0.05$).

Results

Toxicity tests

Toxicity values of pyriproxyfen and methoxyfenozide on *E. kuehniella* larvae are shown in Table 1. ID_{10}, ID_{30} and ID_{50} values of tested chemicals were 0.05, 0.1 and 0.16 µg/mg larvae for pyriproxyfen and 0.23, 0.32 and 0.4 µg/mg larvae for methoxyfenozide. Based on ID_{50} values and estimated relative toxicity, adult emergence inhibition activity of pyriproxyfen was 2.56 times more than that of methoxyfenozide (Table 1). The probit regression of toxicity of pyriproxyfen and methoxyfenozide is shown in Fig. 1. Regression lines of the compounds were not parallel (χ^2 = 29.6, df = 1, P < 0.0001).

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Table 1 Inhibition doses (IDs) of pyriproxyfen and methoxyfenozide for *Ephestia kuehniella.*

Insecticide	*n*	ID values (µg/mg larvae)[1]			Slope ± SE	X^2 (df)	RT (95% CL)[2]
		ID_{10}	ID_{30}	ID_{50}			
Pyriproxyfen	560	0.05 (0.04-0.06)	0.1 (0.09-0.12)	0.16 (0.14-0.18)	2.86 ± 0.22	3.93 (4)	2.56 (1.61-3.43)
Methoxyfenozide	560	0.23 (0.20-0.25)	0.32 (0.30-0.34)	0.40 (0.38-0.43)	5.63 ± 0.45	3.33 (4)	

[1] ID values are expressed as µg/mg larvae with their 95% confidence limits (CL).
[2] Relative toxicity = ID_{50} of methoxyfenozide divided by ID_{50} of pyriproxyfen.

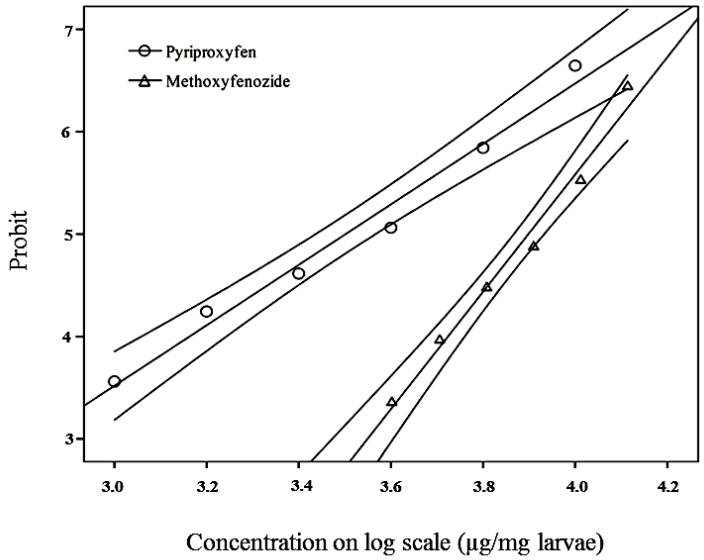

Figure 1 Probit mortality of two-days-old fifth instar larvae of *Ephestia kuehniella* after topically treating with pyriproxyfen and methoxy fenozide.

Effects of chemicals on hemocytes

Time-dependent changes in the total number of hemocytes of two-day-old fifth instar larvae of *E. kuehniella* are shown in Fig. 2. Two tested IGRs had inverse effect on THC. Pyriproxyfen caused a significant reduction in THC in a dose- and time-dependent manner. Total number of hemocytes in the larvae treated with 0.16 µg/mg larvae were reduced to 13730 cell/mm^3 after 24 h compared to control larvae of 33240 cell/mm^3. In contrast, methoxyfenozide caused a noticeable time-dependent increase in THC especially at 0.32 µg/mg larvae so that THC reached to 46200 cell/mm^3compared to 31680 cell/mm^3 in control after 24 h. However, THC showed a reducing trend at 0.4 µg/mg larvae for 24 and 48 h post-treatments.

The effect of pyriproxyfen and methoxyfenozide on DHC in two-day-old fifth instar larvae is shown in Table 2. A significant reduction in prohemocytes ($F = 36.00$; $df = 3$; $P < 0.0001$), plasmatocytes ($F = 242.55$; $df = 3$; $P < 0.0001$) and spherulocytes ($F = 17.63$; $df = 3$; $P < 0.0001$) was observed after 24 h when dose of pyriproxyfen increased from 0.05 to 0.16 µg/mg larvae. In contrast, increasing dose of this pesticide caused a significant increase in granulocytes ($F = 105.71$; $df = 3$; $P < 0.0001$) and partially oenocytoids ($F = 8.29$; $df = 3$; $P < 0.0001$). Also, there was a noticeable decline in mitotically dividing hemocytes (MI) in all doses of pyriproxyfen ($F = 25.32$; $df = 3$; $P < 0.0001$) (Table 2).

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In case of methoxyfenozide, increasing dose resulted in significant increase in plasmatocytes ($F = 62.70$; $df = 3$; $P < 0.0001$), oenocytoids ($F = 14.08$; $df = 3$; $P < 0.0001$) and MI ($F = 4.53$; $df = 3$; $P < 0.01$). However, a declining trend was recorded in prohemocytes ($F = 98.96$; $df = 3$; $P < 0.0001$), granulocytes ($F = 48.09$; $df = 3$; $P < 0.0001$) and spherulocytes ($F = 10.21$; $df = 3$; $P < 0.0001$) 24 h after treatment (Table 2).

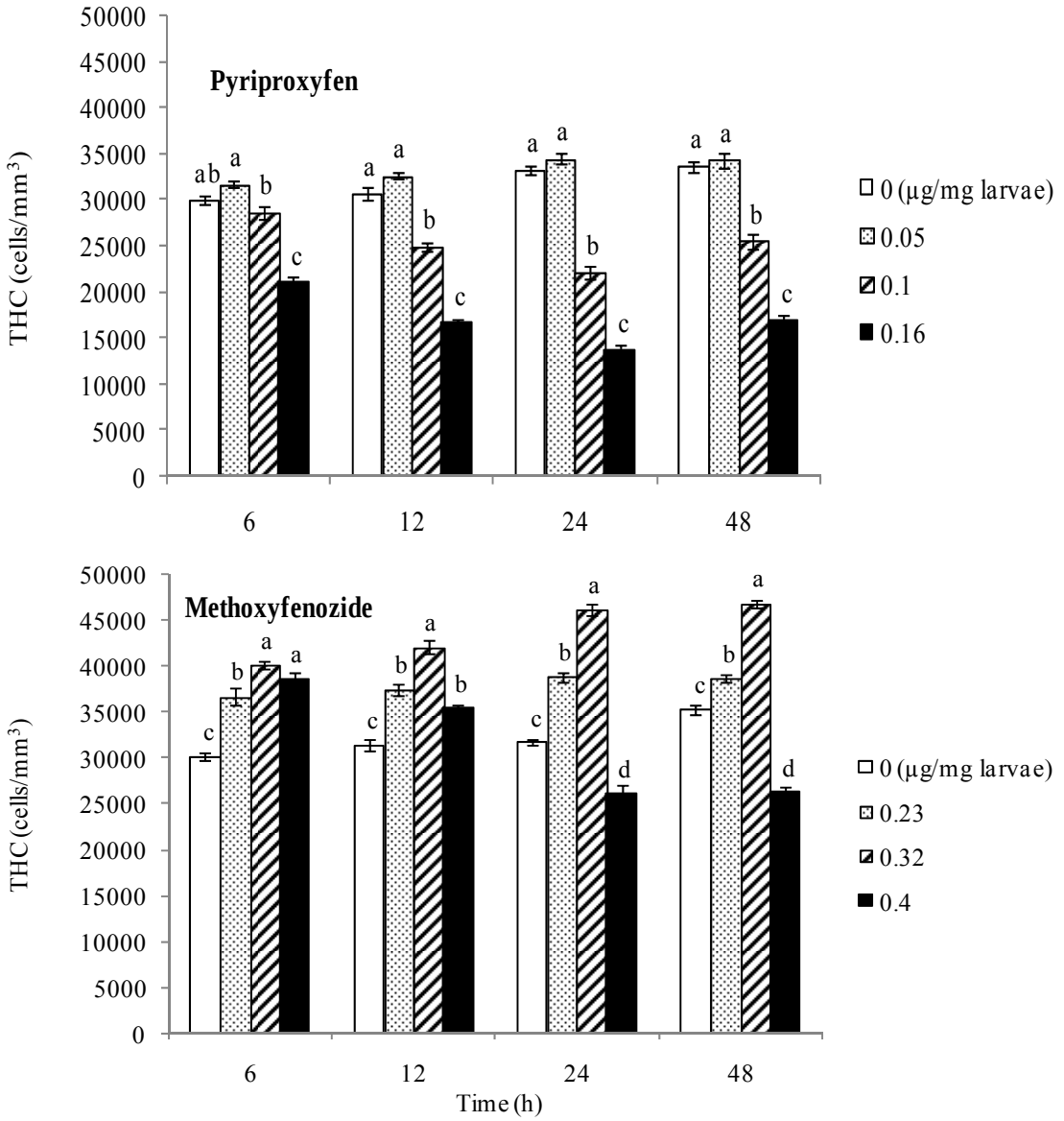

Figure 2 Total hemocyte count (THC/mm^3) in pyriproxyfen and methoxyfenozide topical treatment to two-day-old fifth instar larvae of *Ephestia kuehniella*, 6 to 48 h post-treatment. Values in each time interval followed by the same letters are not significantly different (Tukey test, $p < 0.05$).

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Table 2 Differential hemocyte count (DHC) in larvae of *Ephestia kuehniella* treated with pyriproxyfen and methoxyfenozide after 24 h.

Insecticide	Dose (µg/mg larvae)	% Hemocyte type (Mean ± SE)[1, 2]					
		PR	PL	GR	OE	SP	MI
Pyriproxyfen	0	11.62 ± 0.51^{a}	47.40 ± 1.87^{a}	31.58 ± 0.78^{c}	3.50 ± 0.57^{b}	5.90 ± 1.18^{a}	4.50 ± 0.62^{a}
	0.05	10.50 ± 1.06^{a}	44.80 ± 2.37^{a}	34.30 ± 1.50^{c}	6.50 ± 0.83^{a}	3.90 ± 0.43^{b}	1.80 ± 0.25^{b}
	0.10	7.30 ± 0.53^{b}	31.20 ± 0.80^{b}	51.50 ± 2.6^{b}	7.20 ± 0.51^{a}	2.80 ± 0.48^{bc}	1.50 ± 0.35^{b}
	0.16	4.20 ± 0.33^{c}	26.50 ± 3.50^{c}	60.80 ± 1.52^{a}	6.70 ± 0.25^{a}	1.80 ± 0.48^{c}	0.70 ± 0.43^{b}
Methoxyfenozide	0	15.00 ± 0.54^{a}	41.50 ± 2.90^{c}	31.00 ± 1.83^{a}	7.20 ± 0.58^{b}	5.30 ± 0.30^{a}	4.20 ± 0.48^{b}
	0.23	10.10 ± 0.36^{b}	48.90 ± 1.73^{b}	25.80 ± 0.66^{b}	12.10 ± 1.71^{a}	3.10 ± 0.24^{b}	7.20 ± 0.34^{a}
	0.32	5.30 ± 0.37^{c}	58.30 ± 1.09^{a}	22.20 ± 0.68^{c}	11.20 ± 0.95^{a}	3.00 ± 0.54^{b}	7.60 ± 1.01^{a}
	0.40	6.60 ± 0.43^{c}	60.20 ± 3.34^{a}	20.70 ± 0.37^{c}	10.00 ± 0.52^{a}	2.50 ± 0.54^{b}	6.90 ± 0.48^{a}

[1] Means followed by the same letters in each column are not significantly different (Tukey's test, $p < 0.05$).
[2] PR, prohemocyte; PL, plasmatocyte; GR, granulocyte; OE, oenocytoid; SP, spherulocyte; MI, mitotic index.

Discussion

Our previous study indicated five main types of circulating hemocytes in *E. kuehniella i.e.*the prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), oenocytoids (OEs) and spherulocytes (SPs) (Ghasemi *et al*., 2013).The hematological data showed changes in the total and differential counts of hemocytes when treated with pyriproxyfen and methoxyfenozide. In general, total number of hemocytes is known to change in association with both of detoxification and immune defenses (Kurihara *et al*., 1992), so it is not surprising that both pyriproxyfen and methoxyfenozide affect hemocyte abundance and variation. The findings indicated that hematological effects of tested IGRs were in opposite directions. It is believed that in insects, THC is dependent to ecdysone titer (Prasada Rao *et al*., 1984). Also, Akai and Sato (1971) reported that the increase in ecdysteroids level in hemolymph of *Bombyx mori* L. resulted in violent release of hemocytes from the hematopoietic organs. Nakahara *et al*. (2003) verified that these hemocytes consisted primarily of prohemocytes and plasmatocytes. Similar results were also reported for other moths such as *Pseudoplusia includens* (Walker) and *Spodoptera frugiperda* (Smith) (Gardiner and Strand, 2000), and *Manduca sexta* (L.) (Nardi *et al*., 2003). These results indicate the importance of ecdysteroids titer in the hemocyte production of insect. So, it could be concluded that increase in total number of hemocytes of methoxyfenozide-treated larvae may be due to stimulation of hemocyte release from hematopoietic organs as well as increase in mitotic activity of hemocytes. By contrast, reduction in THC of pyriproxyfen-treated larvae could be linked to interference of pyriproxyfen on ecdysone biosynthesis. Results of the present study show that all circulating hemocyte types, especially plasmatocytes and granulocytes, were affected by the tratment of tested IGRs. In case of the PRs, a declining trend was recorded with increase in dose of both compounds. Pandey *et al*. (2012) pointed out that the reduction in PRs is probably linked to the inhibition of their production from hematopoetic organs or to their conversion to other hemocyte types. It was found that treatment of *E. kuehniella* larvae with pyriproxifen and methoxyfenozide affected the number of PLs and GRs. Results show that the number of GRs increased as the dose of pyriproxifen increased, meanwhile, a declining

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trend was observed in the number of PLs. In contrast, methoxyfenozide had opposite effects on PLs and GRs so that treatment of larvae with this IGR caused increase in proportion of PLs but reduction of GRs. Since PLs are considered as the main hemocyte type active in the cellular immunity of lepidopterous insects (Strand, 2008), it seems that pyriproxifen acts as an immunosuppressive compound by reducing number of PLs. These findings are in agreement with the results reported by Kwon and Kim (2007) who observed immunosuppressive action of pyriproxifen in *Bt*-infected *P. xylostella*. OEs are also found to increase in response to tested IGRs. These hemocyte types have crucial roles in phenoloxidase (PO) cascade when an immune challenge occurs (Beckage, 2008; Strand, 2008). So, increase in the number of OEs in treated larvae might be attributed to their response to IGRs.

In conclusion, the present study demonstrated the hematological effects of pyriproxifen and methoxyfenozide on the *E. kuehniella* larvae. Our findings revealed the appropriate growth regulating activity and immunosuppressive action of the JH agonist, pyriproxifen, against tested species.

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تأثیر پیری‌پروکسیفن ومتوکسی‌فنوزاید روی سلول‌های خونی شب‌پره مدیترانه‌ای آرد *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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چکیده: شب‌پره مدیترانه‌ای آرد *Ephestia kuehniella* Zell. یکی از آفات اصلی محصولات انباری در سرتاسر دنیا می‌باشد. مشکلات متعدد مربوط به استفاده از حشره‌کش‌های متداول، نیاز برای توسعه ترکیبات جایگزین ایمن مثل تنظیم‌کننده‌های رشد حشرات را به‌شدت توجیه می‌کند. در مطالعه حاضر، فعالیت تنظیم‌کنندگی رشد و اثرات هماتولوژیک پیری‌پروکسیفن و متوکسی‌فنوزاید روی لاروهای شب‌پره آرد ارزیابی شد. در آزمایش‌های مرحله اول، سمیت موضعی دو تنظیم‌کننده رشد در شرایط آزمایشگاهی مورد بررسی قرار گرفت. نتایج آزمایش‌های زیست‌سنجی نشان داد که پیری‌پروکسیفن با دز ممانعت‌کننده از ظهور ۵۰ درصد جمعیت (ID_{50}) معادل ۰/۱۶ میکروگرم بر میلی‌گرم لارو و متوکسی‌فنوزاید با ID_{50} معادل ۰/۴ میکروگرم بر میلی‌گرم لارو اثر تنظیم‌کنندگی رشد قابل‌ملاحظه‌ای روی لاروهای دو روزه سن پنجم شب‌پره آرد دارند. در آزمایش‌های مرحله دوم، اثر دزهای برآورد شده آفت‌کش‌ها روی خصوصیات هماتولوژی گونه مورد آزمایش شامل تعداد کل سلول‌های خونی و تعداد تفرقی آن‌ها مورد بررسی قرار گرفت. نتایج حاصل نشان داد که تیمار کردن لاروهای شب‌پره آرد با آفت کش‌های مورد بررسی به‌طور معنی‌داری تعداد کل و تفرقی سلول‌های خونی را کاهش می‌دهد. تعداد کل سلول‌های خونی و نسبت پلاسماتوسیت‌ها با افزایش دز پیری‌پروکسیفن کاهش یافت. درحالی‌که، جمعیت گرانولوسیت‌ها در لاروهای تیمار شده با پیری‌پروکسیفن افزایش پیدا کرد. برعکس، دزهای زیرکشنده متوکسی‌فنوزاید باعث افزایش قابل ملاحظه تعداد کل سلول‌های خونی و جمعیت پلاسماتوسیت‌ها شد اما، درصد گرانولوسیت‌ها کاهش یافت. چون تعداد کل سلول‌های خونی و نسبت پلاسماتوسیت‌ها در واکنش‌های ایمنی حشرات بسیار مهم هستند بنابراین، پیری پروکسیفن می‌تواند به‌عنوان یک آفت‌کش بازدارنده سیستم ایمنی، در کنترل تلفیقی شب‌پره آرد مورد استفاده قرار گیرد.

واژگان کلیدی: سموم تنظیم‌کننده رشد، تعداد کل سلول‌های خونی، تعداد تفرقی سلول‌های خونی، پلاسماتوسیت، گرانولوسیت

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