JOURNAL OF ENTOMOLOGICAL SOCIETY OF IRAN
2018, 37(4), SUPPLEMENTARY, 515–525
نامه انجمن حشره‌شناسی ایران ۵۲۵– ۵۱۵ ضمیمه ,(٤)۳۷ ,۱۳۹٦

DOI: 10.22117/jesi.2018.116103

SPECIAL ISSUE: PROCEEDINGS OF THE 2ND IRANIAN INTERNATIONAL CONGRESS OF ENTOMOLOGY

The effect of thermal stresses on the immune system of the potato tuber moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae)

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Abstract
The hemocytes of insects are one of important components of immune system of insects against various stresses such as pathogens attack, parasitoids, starvation period and temperature changes. Hemocytes characteristics recognition and frequency in cellular immune studies will help us in order to better pest control. In this study hemocytes of fourth instar larvae of potato tuber moth *Phthorimaea operculella* (Zeller) were identified after staining with Giemsa and by light microscopy at 40x magnification. Five types of identified hemocytes were prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), oenocytoids (OEs) and spherulocytes (SPs). The effect of different thermal stresses was also investigated for 24 hours on cellular defense of fourth instar larvae. In addition number of various hemocytes and total number of blood cells were investigated. At 35 °C, total hemocyte count (THC) and PLs of larvae was increased significantly compared to the control (25±1 °C). Also, chill stress (4 °C) showed a significant decrease in THC, PLs and OEs compared to the control. These findings could be used as a base for further investigation on the immunology studies of potato tuber moth.
Key words: *Phthorimaea operculella*, hemocytes, Cellular defense, heat and chill stresses, Total Hemocyte Count.

بررسی تاثیر تنش‌های دمایی روی سامانه ایمنی بید سیب‌زمینی

Phthorimaea operculella **(Lepidoptera: Gelechiidae)**

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چکیده
سلول‌های خونی حشرات، یکی از اجزای مهم سیستم ایمنی حشرات در برابر انواع تنش‌ها مانند حمله بیمارگرها، پارازیتوئیدها، دوره گرسنگی و تغییرات دما می‌باشند. شناخت ویژگی‌های سلول‌های خونی و فراوانی آن‌ها در مطالعات ایمنی‌شناسی سلولی در جهت کنترل بهتر آفت به ما کمک خواهد کرد. در این تحقیق سلول‌های خونی لارو سن چهار *Phthorimaea operculella* (Zeller) پس از رنگ‌آمیزی با Giemsa با میکروسکوپ نوری و بزرگ‌نمایی ٤٠ شناسایی شدند. ۵ نوع هموسیت در حشره بید سیب‌زمینی شناسایی شد که شامل پروهموسیت‌ها، پلاسموتوسیت‌ها، گرانولوسیت‌ها، اونوسیتوئیدها و اسفرولوسیت‌ها بودند. اثر تنش‌های دمایی مختلف نیز به مدت ٢٤ ساعت بر دفاع سلولی لاروهای سن چهارم بید سیب‌زمینی بررسی شد. به علاوه تعداد هموسیت‌های مختلف و تعداد کل سلول‌های خونی بررسی شدند. نتایج نشان داد که تعداد کل هموسیت‌ها و پلاسموتوسیت‌های لاروهایی که به مدت ٢٤ ساعت تحت تنش دمایی ۳۵ درجه سانتی‌گراد قرار گرفتند، نسبت به شاهد (دمای ۱±۲۵ درجه سانتی گراد) به طور معنی‌داری افزایش یافت. همچنین در اثر تنش سرما (دمای ٤ درجه سانتی گراد)، تعداد کل هموسیت‌ها، پلاسموتوسیت‌ها و اونوسیتوئیدها نسبت به شاهد کاهش معنی‌داری نشان داد. این یافته‌ها می‌تواند به عنوان مقدمه‌ای برای تحقیقات بیشتر در راستای مطالعات ایمنی‌شناسی بید سیب‌زمینی مورد استفاده قرار گیرد.
واژگان کلیدی: بید سیب‌زمینی، سلول‌های خونی، دفاع سلولی، تنش‌های سرمایی و گرمایی، تعداد کل سلول‌های خونی.

دریافت: ۱۳۹٦/۸/۳۰، پذیرش: ۱۳۹٦/۹/۱۸.

Received: 21 November 2017, *Accepted:* 9 December 2017
Subject Editor: Saeid Moharramipour

Introduction

The potato tuber moth, *Phthorimaea operculella* (Zeller) is an oligophagous pest of solanaceous plants including potatoes, tomatoes, tobacco, eggplant, peppers, okra and nightshade and widely has been distributed in tropical and subtropical regions (Fenemore, 1988). The larvae of this pest bore tunnels in leaves, stem, petiole and potato tubers and main damage is to dig tunnels in potato tubers. But in tropical and subtropical areas of the field on the leaves of the host plant also creates considerable damage. In storages infected tubers may reduce the marketability and damage the tubers in storage, especially in storages without cooling system can be very severe (Arnone *et al.,* 1998). This pest reduces product quality and increases risk of infection to fungal and bacterial pathogens. The pest attack to the aerial parts and tubers also can reduce significantly yields of potatoes (Capinera, 2001). Experience has proved that chemical control of this pest due to hiding in the leaves, stems and tubers and rapid development of resistance to the insecticides alone are not enough and have to use different methods of cultural, mechanical, biological and chemical deal with this pest (Bacon *et al.,* 1972). Due to the undesirable economic impact of the potato tuber moth and the need to control it, hemocytes accurate identification of this pest and reactions of cellular defense against chemical compounds, contaminant, spore of fungal pathogens and environmental stresses such as temperature will help us in order to better pest control.

Based on morphological characteristics, function and tissue chemistry, several types of hemocytes have been identified in insects (Gupta, 1985; Brehélin *et al.,* 1989). In the hemolymph of fifth instar larvae of silkworm *Bombyx mori* (Linnaeus), 5 types of blood cells is detected (Akai & Sato, 1973). Similar results were also reported for other lepidopterous larvae, such as *Plutella xylostella* (Linnaeus) (Huang *et al.,* 2010), *Ectomoyelois ceratoniae* (Zeller) (Khosravi *et al.,* 2012), *Hyphantria cunea* (Drury) (Ajamhassani *et al.,* 2013) and *B. mori* (Tan *et al.,* 2013). Studies on the effect of temperature on the hemocytes count of insects, have been conducted by researchers, indicate different results. Some emphasized that the low temperatures reduce total hemocyte count (THC) in insects (Tauber & Yeager, 1935; Tiwari & Shukla, 2000) and some believed that the high temperatures increase THC (Tauber & Yeager, 1935; Rosenberger & Jones, 1960). In some cases there was no change in the THC, in low temperatures (Rosenberger & Jones, 1960). Moreover, very little and contradictory information is available on the effect of temperature on the differential hemocyte count (DHC) (Arnold, 1952; Behera *et al.,* 1999; Pandey *et al.,* 2003). It is proved that there is a close relationship between the growth and development of insects with the temperature. In some of insect species when temperature increases, growth rate also increases and generation time will be short (Wigglesworth, 1972; Kiuchi *et al.,* 2008). In this regard the effect of temperature on the number and frequency of hemocytes in different developmental stages has been conducted (Lackie, 1988; Gardiner & Strand, 2000; Pandey *et al.,* 2003; Kiuchi *et al.,* 2008). Reporting of Pandey and colleagues in 2010 were expressed in relation to the impact of temperature on the shape and number of lepidopteran blood cells. For example, heat stress causes a significant change in blood cell immune responses of tropical silkworm *Antheraea mylitta* D. In this study, DHC changes, under the influence of various thermal stresses was remarkable. The number of PRs and PLs decreased under the influence of chill stress, while a brief increase in the number of GRs, SPs, adipohemocyte and OEs was observed. In the larvae of under heat stress, the amount of PRs, PLs and OEs increased, while other hemocytes decreased. Short term temperatures (50°C for 1 hour), showed different patterns in the relative percentages of different types of hemocytes. Exposure to heat for a short period caused damage to the cells under different conditions. Pandey et al. (2010) reported that PLs and GRs are the only group that they are always more impressible of other hemocytes under different temperature regimes. The impact of heat stresses on the shape of hemocytes of the larvae of *A. mylitta* showed that heat stress leads to clutter cells shape. So that when the larvae exposed at the temperature of 50 °C for 1 hour, reactions such as loss of cytoplasmic compactness of pseudopods in PLs, vacuolization in PLs and GRs and nuclear fragmentation occurred in PRs that even led to cell death in some cases (Pandey *et al.,* 2010). In another study on the larva of *Danaus chrysippus* L. showed that chill stress causes decreasing of blood cell counts, however heat stress led to increasing of blood cells (Pandey *et al.,* 2008b). The immune responses of *Ephestia kuehniella* Zell, also were assessed against thermal stresses in another research. According to the observations, the shape of hemocytes and their numbers drastically changed under the influence of high temperature stresses (Ghasemi *et al.,* 2013). PLs and GRs cell wall, as the important hemocytes involved in cellular immune were torn at the temperature about 40 °C and their cell contents were released into the hemolymph (Ghasemi *et al.,* 2013). Jones (1967a) in research of THC changes during developmental stage larvae of *Galleria mellonella* L. achieved to this result that fixed and non-fixed heat both of them cause to increasing of THC, although the its amount in insects that exposed under fixed heat, was significantly higher. He suggested that this increase in the number of cell is possibly because of dehydration due to drying of it in heat effect (Jones, 1967a). In study of Tauber and Yeager in 1935, the rate of mitosis division of *Blaberus* sp hemocytes that exposed at the temperature of 37 °C, increased (Tauber & Yeager, 1935). Studies on *D. chrysippus* have shown that there are 6 types hemocytes in the hemolymph of this lepidopteran (Ribeiro & Brehelin, 2006). These hemocytes reveal undesirable impacts of stresses (chill and heat), that may be similar to the stress caused by the hemolymph toxicity of the bug of *Dysdercus koenigi* (Fabr) from order of Hemiptera (Tiwari *et al.,* 2006). Hemocytes of this lepidopteran sensitive to temperature and it seems PRs are the most sensitive hemocytes than other cells, which generally are responsible for the natural physiological function of blood as stem cells (Pandey *et al.,* 2008a).

Materials and methods

Insect Rearing

The early population of potato tuber moth to form colony, were collected of infected tubers available at Square Farmer's Market of Kashmar city. Colony of potato tuber moth were set into box of cubes rectangular tin like with height of 40 cm and aperture dimensions 30 × 30 cm that containing potato tubers. So that the aperture of the box was covered by isinglass talc and just one side of it was covered by a thick cloth sleeve like that it was possible access to tubers and insects for transfer them. Tubers were reviewed daily and when were used by the larvae or were decayed, were replaced with fresh tubers. When the adult insects appeared, very small drops of honey was rubbed on the inner surface of the rearing box that adult moth feed on it. Insect rearing in growth chamber with temperature of 24±1 °C, relative humidity 45±5% and 14:10 L:D was done.

Identify blood cells of *P. operculella* by light microscopy

In order to identify blood cells, the hemolymph of 10 larvae (10 replications) were collected. At the first amputate the foreleg of larva with scalpel and amount of 2 µl of the hemolymph placed on a slide and a smear was prepared on each slide with the edge of another glass slide. After drying, smears were stained with amount of Giemsa (solution 9: 1 Giemsa and distilled water) for 20 min and washed with distilled water. Slides were then washed in water and rinsed in saturated lithium carbonate for 10 seconds and finally washed with distilled water, and dried. A permanent slide was prepared by Canada balsam and sorts of them identified according to the available sources (Yeager, 1945). To view and detect blood cells, an optical microscope with a magnification of 40 was used.

Total hemocyte count (THC)

For THC, the hemolymph of 10 larvae of 2, 3 and 4 instars separately collected by using a micropipette and were diluted with physiologic buffer. Anticoagulant buffer used in the test was Tyson solution (Mahmood & Yousaf, 1985). THC was conducted with a standard Neubauer hemocytometer. The cells were counted using a light microscope at 40x magnification and number of total hemocytes per cubic millimeter (mm3) was calculated using the following formula of Jones (1962):

$$\frac{\text{Hemocytes in} \times 1\text{mm}^2 \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{No. of squares counted}}$$

Effect of different thermal stresses on THC, PL, GR, PR and OE

To this end, 20 larvae of fourth instar of the potato tuber moth were used. Treatments include infected tubers by *P. operculella* that exposed at the two temperatures 4 °C and 35°C for 24 hours. Control treatment also was the infected tubers that exposed at the temperature 25±1 °C. Total hemocyte count, PLs, GRs, PRs and OEs of treatment larvae were counted. Data analysis was performed with SAS program and comparison of means by Tukey's test ($p<0.01$) was conducted.

Results

The investigation of cytology, THC and various hemocytes of 2, 3 and 4 instars larvae

By using a light microscopy, hemocytes of 2, 3 and 4 instars larvae of *P. operculella* identified. These hemocytes are PRs, PLs, GRs, OEs and SPs.

PRs were observed perfectly round and were the smallest hemocytes with central nucleus (Fig.1). These cells after the SPs had the lowest abundance than the other hemocytes. The number of these cells in fourth instar larvae were significantly more than the 3 and 2 instars larvae ($F=8.34$, $df=2$, $p\leq0.0015$) (Table 1).

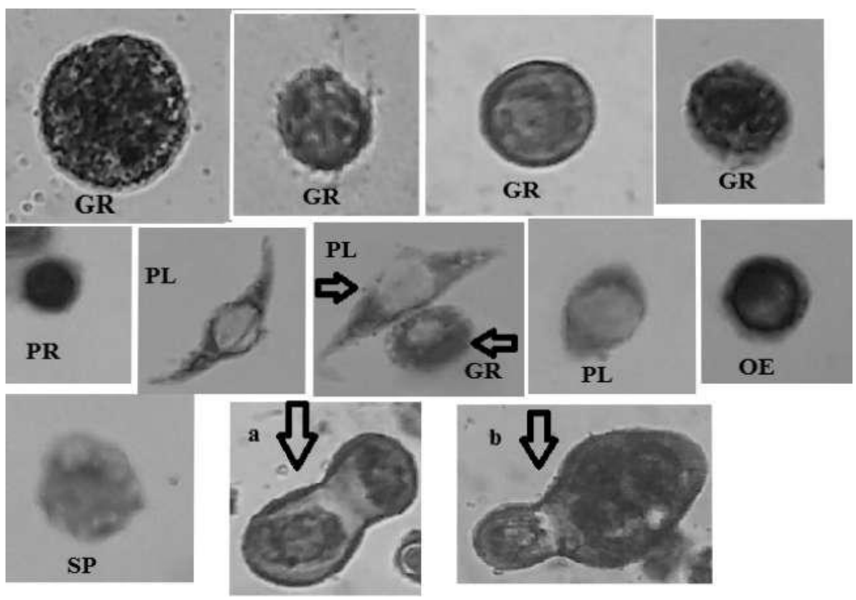

Fig. 1. Light microscopy (at 40x magnification) pictures of *P. operculella* hemocytes stained with Giemsa. GR= Granulocyte, PL= Plasmatocyte, PR= Prohemocyte, OE= Oenocytoid, SP= Spherulocyte, a and b= Mitosis divisions in Granulocytes

PLs were spindle-shaped with two cytoplasmic papillae on either side of cell and sometimes without cytoplasmic papillae. These cells had polymorphic profile in the larvae of *P. operculella* and were observed in various sizes (Fig.1). PLs after the GRs had the most abundance in this insect. These cells in 4 and 3 instars larvae of *P. operculella* were significantly more than the second instars larvae ($F=9.98$, $df=2$, $p\leq0.0006$) (Table 1).

GRs were round or oval cells that were larger than PRs and their cytoplasmic surface had small granules. GRs size was varied from small to large cells which were observed in insect's hemolymph (Fig.1). These hemocytes had the most abundance in hemolymph of this insect. These cells in 4 and 3 instars larvae of *P. operculella* were significantly more than the second instars larvae ($F=37.33$, $df=2$, $p\leq0.0001$) (Table 1).

Table 1- Total Hemocyte count and various hemocytes of 10 larvae of 2nd, 3rd and 4th instars in *P. operculella*

Phase	PR	PL	GR	OE	SP	THC
L2	28.90±5.1b	205.70±30.24b	530.3±45.98b	56.10±8.79a	8.500±3.80a	831.3±82.52b
L3	23.80±4.53b	498.10±61.71a	1283.5±114.72a	57.80±5.77a	0.000±0a	1861.5±140.39a
L4	62.90±10.76a	428.40±47.80a	1725.5±118.62a	81.60±9.74a	0.000±0a	2293.3±139.11a

Columns with same letter were not significantly different at p<0.01, Tukey's test

OEs were observed circular with a lateral nucleus (Fig.1). These hemocytes had lower abundance than GRs and PLs. But significant difference were not found in the number of OEs in 2, 3 and 4 instars larvae ($F=2.96$, $df=2$, $p\leq0.0687$) (Table 1).

SPs or spherule cells were observed with a compact nucleus that cytoplasmic surface of these cells had several spherules (Fig.1). These cells had the lowest abundance of hemocytes. These cells were observed very few in second instars larvae so that there had no significant difference with 3 and 4 instars larvae ($F=5.00$, $df=2$, $p\leq0.0142$) (Table 1).

THC also showed a direct correlation with increase in the larval instar. The most THC related to the fourth instar larva and the lowest THC were observed in second instar larva, so that THC in 3 and 4 instars larvae had significant difference than THC of the first instar larva ($F=36.90$, $df=2$, $p\leq0.0001$) (Table 1).

Effect of different thermal stresses on THC and various hemocytes of fourth instar larva

The results of the effects of different thermal stress on hemocytes of fourth instar larva of *P. operculella* are presented in Table 2. As it can be seen, the larvae exposed to 35 °C for 24 hours; THC, GRs, PLs and OEs compared to the control temperature (25±1 °C) increased so that significant changes were created in THC ($F=5.24$, $df=2$, $p\leq0.0082$) and PLs ($F=13.47$, $df=2$, $p\leq0.0001$) compared to the control (Table 2). Also in the effect of chill stress (4 °C), THC, GRs, PLs, PRs and OEs compared to the control temperature (25±1 °C) decreased so that significant changes were created in THC ($F=5.24$, $df=2$, $p\leq0.0082$), PLs ($F=13.47$, $df=2$, $p\leq0.0001$) and OEs ($F=12.24$, $df=2$, $p\leq0.0001$) compared to the control (Table 2).

Table 2- Effect of different temperatures on total hemocyte count and various hemocytes of 20 larvae of fourth instar in *P. operculella*

Temperature	PR	PL	GR	OE	THC
25±1°C	61.20±9.42a	420.75±37.64ab	1522.4±105.96a	86.70±12.01a	2083.4±143.65ab
35°C	56.95±6.32a	538.90±34.35a	1664.4±84.41a	92.65±10.42a	2313.0±119.68a
4°C	59.50±5.85a	272.85±36.88b	1343.9±113.59a	32.30±4.25b	1708.5±135.56b

Columns with same letter were not significantly different at p<0.01, Tukey's test

Discussion

The present study provides detailed information of hemocyte profile and hemogram of the potato tuber moth, *P. operculella*. In 1995, the lepidopteran hemocytes were divided into five classes on the basis of morphology, i.e. prohemocytes, plasmatocytes, granulocytes, oenocytoids and spherulocytes (Strand & Pech, 1995). In this study also all of these five morphotypes of hemocytes in *P. operculella* larvae were observed. Differential hemocyte count showed that GRs and PLs together had about 95% abundant of total hemocyte count of fourth instar larvae of the potato tuber moth. In fact, PLs and GRs that known as immunocyte had most activity in the processes of cellular defense. Prior studies have shown that PLs and GRs were responsible as cellular immune responses in many lepidopteran insect larvae, such as *G. mellonella* (Tojo *et al.*, 2000) and *Manduca sexta* L. (Ling & Yu, 2006) and together usually comprise more than 50 % of the hemocytes in circulation (Lackie, 1988; Ratcliffe, 1993). According to the results of cell measurements, it is shown that each cell morphotype has of a varied size. In this study also various forms of some hemocytes and especially GRs was found that size of them were varied from small to large cells which scattered in insects hemolymph. This variation in the form and size has brought many problems in the classification of hemocytes because the insect blood cells are highly polymorphic, and the particular form they present at any one time seems to depend on the age, developmental stage, nutritional state and species of insect as well as on the methods of collection and examination used by the investigator (Jones, 1962; Lai-Fook & Neuwirth, 1972). The maintenance of circulating hemocytes in larval Lepidoptera has been attributed to both the release of hemocytes from hematopoietic organs and the mitosis of hemocytes in the circulatory system (Gardiner & Strand 2000). The levels of mitotic activity in circulating hemocytes rarely exceed 1 % in almost all cases (Jones, 1967a; Jones & Liu 1968; Jones, 1967b), but it is shown that in *P. operculella* this activity varies with developmental stage of insect and mitosis division types also was seen in high level in the fourth instar larvae which feed on tubers efficiently (Fig.1). In many insect species, fluctuations in the number of hemocytes are influenced by the release of hemocytes from the hemapoietic organ and attachment of the cells to internal tissues (Tu *et al.,* 2002; Okazaki *et al.,* 2006). The number of hemocytes in circulation can change rapidly in response to stresses, such as wounding, infection, starvation, nutrition and temperature changes (Gillespie *et al.,* 2000; Mowlds & Kavanagh 2008). As low and high temperatures are a source of stress for insects, it is possible that the number of hemocytes was directly altered by the change in temperature. In this study the results of the tests clearly indicated that heat stress causes a significant increase in THC and in contrast cold stress showed a significant reduction in THC. So, it seems that exposing the insects to high temperatures can increase the environmental fitness of larvae through a similar mechanism to thermoregulatory behaviour by increasing THC especially PLs. It seems that another possible reason for the increase in THC is due to loss of body fluid as a result of desiccation. On the other hand, PLs count increased significantly in the effect of heat stress and chill stress resulted a significant reduction in PLs count. So it is expected that this cells as the one of important hemocytes involved in cellular immune, play an important role in cellular defense of *P. operculella*.

Conclusion

In this study, we determined the morphological characteristics of hemocytes of *P. operculella* and changes in hemocyte composition during the larval development that were essential for further understanding of cellular responses of this insect due to the undesirable economic impact of the potato tuber moth and the need to control it, hemocytes accurate identification of this pest and reactions of cellular defense against chemical compounds, contaminant, spore of fungal pathogens and environmental stresses such as temperature will help us in order to better pest control. Our findings revealed clearly the impact of thermal stress on shape and number of blood cells and THC of *P. operculella*. However, further investigations may have to be performed to determine if changes in hematological properties of thermal treated larvae could affect their cell mediated immune responses. It might then be important to conduct next experiments on the effect of thermal shocks upon hemocyte composition and different immune responses of *P. operculella* after infection with various pathogens.

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