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Changes in the haemocytes of *Agrotis ipsilon* larvae (Lepidoptera: Noctuidae) in relation to dimilin and *Bacillus thuringiensis* infections

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ABSTRACT

Five types of haemocytes are observed in the fourth larval instar of the black cutworm, *Agrotis ipsilon:* prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherule cells (SPs) and adipohaemocytes (ADs). Infection of *A. ipsilon* fourth larval instar with *Bacillus thuringiensis* and dimilin resulted in a reduction of the total haemocyte count. Changes in the differential haemocyte population during bacterial and dimilin infections have been assessed. The PRs % decreased significantly while SPs, PLs, and GRs % increased significantly after the application of the two insecticides at 12 and 24 h. Ultrastructural alternations and malformations have been observed in circulating haemocytes of *A. ipsilon* larvae treated with dimilin and *B. thuringiensis.*

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1. Introduction

The black cutworm, *Agrotis ipsilon* (Hufnagel) is one of the agricultural pests that infest various crops worldwide. At the local level, it has long been established in Egypt as a major pest of cotton and vegetable plants (Beheedy, 1982). The hematological studies are important in the field of insect physiology because certain vital activities are performed by haemocytes. Insect haemocytes categorized into several types circulating in the haemolymph. Their primary functions are coagulation, phagocytosis, encapsulation, detoxification, and storage and distribution of nutritive materials. Haemocytes have been studied mostly in Lepidoptera, Hymenoptera, Coleoptera, and Diptera (Gupta, 1985). The prohaemocytes are specialized for division, plasmatocytes, specialized for phagocytosis, granulocytes, spherulocytes and oenocytoid, specialized for secretions and storage; and coagulocytes, specialized for clotting (Brehélin and Zachary, 1986). There is an inherent variability of haemocytes within a species depending on the developmental and physiological stages (Sanjayan et al., 1996; Beetz et al., 2008).

Dimilin and *Bacillus thuringiensis* were used to overcome the cellular immune system employed by the differentiation and haemocyte counting from the treated larvae of the black cutworm. A better understanding of these mechanisms may provide us basic data about reasons for success or failure of bacteria and dimilin, an insect growth regulator (IGR), in insect haemolymph and might lead to new highlights in biocontrol or IGRs to control the *A. ipsilon* or at least enable us to improve strategies of using both of them in integrated pest management programme (IPM). The changes in total haemocyte counts (THCs) and differential haemocyte counts (DHCs) of *A. ipsilon* larvae against *B. thuringiensis* and dimilin, are important criteria, determining cellular immune reactions.

The present work generally is primarily concerned to show some portion of broad spectrum through the effects of *B. thuringiensis* and dimilin (diflubenzuron) on the ultrastructural aspects of haemocytes and their total and differential counts.

2. Materials and methods

2.1. Insect

Fourth instar larvae of *A. ipsilon* were used in the present study. Larvae were supplied by Insect Control Department, National Center for Research, Dokki, Egypt. Larvae were maintained in the laboratory of Entomology Department, Faculty of Science, Cairo University at 25 ± 3 °C and 65 ± 5% RH and a photoperiod of 12:12 (L:D) h. Larvae were fed on castor bean leaves (*Ricinus communius*).

2.2. Insecticides

The commercial powder dimilin (diflubenzuron) was purchased from Nems Service Company, Giza, Egypt. For the experimental purposes, a stock solution of 1% concentration was prepared by mixing 1 mg of dimilin to 100 ml of distilled water; two drops of Tween 80 were added. From the stock solution, 0.5, 0.25, 0.125 and 0.06% solutions were prepared.

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The commercial formulation Dipel2x (*B. thuringiensis* subsp. *kurstaki*) was offered from Botany Department, Faculty of Science, Cairo University. Six serial dilutions of the test samples 1000, 500, 250, 125, 62.5 and 31.25 μg/g of bacteria were used.

The assay procedure proposed by Dulmage et al. (1970) was adopted. Dimilin and bacterial concentrations were applied to fresh castor leaf discs, 1 cm^2 diameter, by dipping castor leaf discs in the experimental solutions for 5 min and let them dry in room temperature for 30 min then allow larvae to feed. Larvae were fed on the treated leaves for 24 h then transferred to feed on untreated leaves for another 72 h. Five replicates, 30 newly emerged fourth instar/replicate, were used for each treatment. Control experiment was also run using larvae feed on leaves treated with distilled water plus two drops of Tween 80. Data on mortalities were recorded using Abbott's formula (1925). The LC_{50} concentrations of dimilin and bacteria were calculated to select and study their effect on the haemocyte count and the ultrastructure of *A. ipsilon* haemocytes.

2.3. Collection and processing of haemolymph

Haemolymph was aseptically collected from chilled, surface sterilized (70% ethanol) larvae by cutting the last larval prolegs. Haemolymph was collected after 12, 24, and 48 h of treatments in chilled sterile tubes. The method of Brehélin et al. (1989) modified by Boucias et al. (1994) was adopted.

2.4. Studies of haemocytes

For total haemocyte counts (THCs), fresh haemolymph was diluted 10-fold with a cold physiological saline buffer containing 0.4% trypan blue (Horohov and Dunn, 1982). Cells in the diluted haemolymph were counted using a Thoma haemocytometer filled with anticoagulant buffer pH 7, TPS/Ca^{2+} (0.075 M Tris, 0.01 M $CaCl_2$, 0.074 M NaCl) (Leonard et al., 1985) under phase contrast optics as described by Arnold (1979). The THC was estimated according to the formula suggested by Jones (1962). The differential haemocyte counts (DHCs) were estimated according to the technique used by Abu El-Magd and El-Kifl (1993) using fresh slide preparations. The slide was examined under differential interference contrast optics, Ziess microscope.

2.5. Electron microscopy (EM)

For EM studies, haemolymph was diluted (1: 1) with a cold physiological saline buffer containing 6% (v/v) glutaraldehyde and chilled for 30 min (Horohov and Dunn, 1982). The fixed cells were centrifuged (6000 rpm, 2 min), the pellet suspended in 0.1 M cacodylate buffer, pH 6.5, containing 2% osmium tetroxide and the mixture incubated for 2 h at 4 °C. The post-fixed cells were washed with distilled water and stained overnight with 0.5% (w/v) uranyl acetate, dehydrated, and embedded in Epon 812 (Luft, 1961). Ultrathin sections were cut using MT2-B ultramicrotome, stained with lead citrate (Reynolds, 1963) for 15 min and examined using a Philips CH 100 electron microscope at 70 kV accelerating voltage.

2.6. Statistical analysis

The data obtained were statistically analyzed using comparison between means of treated and non-treated groups using computer software program, carried out by one-way ANOVA followed by post hoc version 14 using SPSS (analysis with LSD test).

3. Results and discussion

The LC_{50} of dimilin and *B. thuringiensis*, 2.1 μg/ml and 276.5 μg/g, respectively, were selected to study their effect on *A. ipsilon* haemocytes.

3.1. Haemocyte counts

The total number of circulating haemocytes in an insect varies with developmental and physiological stages (Sanjayan et al., 1996; Beetz et al., 2008). Five primary types of haemocytes were observed in the haemolymph of the *A. ipsilon* fourth instar. There were prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherule cells (SPs), and adipohaemocytes (ADs). GRs were the most abundant haemocytes followed by SPs and PLs. ADs were numerically less abundant.

3.1.1. B. thuringiensis

The dynamics of reactions of *A. ipsilon* fourth larval instar against *B. thuringiensis* revealed highly prominent changes in the total haemocyte count (THC). A marked decrease in THC was noted. Quantitative analysis of THC of insects infected with *B. thuringiensis* gave the results shown in Table 1. Bacterial infection has lead to significant decrease in THCs after 12, 24, and 48 h post-infection reaching 12 206.2, 12 797.5, and 11 477.5 cell/mm^3, respectively, while in control THCs were 20 849.0, 23 162.5, and 20 832.5 cell/mm^3, respectively ($p \leq 0.05$). In other words, infection of *A. ipsilon* with *B. thuringiensis* decreases the THCs by 41.5, 44.7, and 44.9% as compared to the control after 12, 24, and 48 h post-infection, respectively. THC decreased in a time-independent manner. Similar results were reported by Ericsson et al. (2009) who studied the immune response to *B. thuringiensis kurstaki* (Btk) in susceptible (Bt-RS) and resistant (Bt-R) *Trichoplusia ni* after exposure to low doses of Btk. They reported a reduction in haemocyte counts after exposure to Btk and after injection with *Escherichia coli*. Also, Johnson et al. (1981) investigated that haemocytes were lost from the circulation by their incorporation into aggregations or by lysis of individual cells after infection of *Homarus amrericanus* with bacteria. Also, the present data agree with those obtained for *Spodoptera littoralis* and *A. ipsilon* larvae infected with *Heterorhabditis heliothidis* nematode (Abu El-Magd and El-Kifl, 1993). On the other hand, the results obtained are in contrast to those detected by De Azambuja et al. (1991) who found that inoculation of *Enterobacter cloacae* caused an immediate sharp increase in the THC of *Rhodnius prolixus* up to day 7 which was followed by a decline of THC after this time. Also, Horohov and Dunn (1982) recorded a marked increase in THC of *Manduca sexta* larvae injected with *Pseudomonas aeruginosa*

Table 1
Total haemocyte counts (THCs) cell/mm^3 of the fourth larval instar *A. ipsilon* following infection with LC_{50} of *B. thuringiensis* and Dimilin.

Time Post-infection (h)	Average no. haemocytes/mm^3		
	Control	*B. thuringiensis*	Dimilin
12	$20\,849.0 \pm 19.13^a$	$12\,206.2 \pm 123.90^b$	$10\,265.0 \pm 99.27^b$
24	$23\,162.5 \pm 16.52^a$	$12\,797.5 \pm 4.797^b$	$11\,437.5 \pm 202.78^b$
48	$20\,832.5 \pm 50.56^a$	$11\,477.5 \pm 15.48^b$	$15\,184.17 \pm 258.11^c$

The different letter(s) are significantly different at $p \leq 0.05$, according to the LSD test.

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