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Effect of *Bacillus thuringiensis* and Farnesol on Haemocytes Response and Lysozymal Activity of the Black Cut Worm *Agrotis ipsilon* Larvae

Hanan H. Awad
Department of Entomology, Faculty of Science, Cairo University, Egypt

Corresponding Author: Hanan H. Awad, Department of Entomology, Faculty of Science, Cairo University, Egypt Tel: +20 105690494 Fax: +20 235728843

ABSTRACT

The aim of the present study was to follow up the defense reaction of the haemocytes from larvae treated with bacteria and farnesol. The lysozymes are a powerful tool of the humoral defense mechanism. The insecticidal activity of *Bacillus thuringiensis* and farnesol on the 4th larval instar of *Agrotis ipsilon* showed a significant dose and time-dependent increase in mortality. The haemocyte population after bacteria and farnesol treatments was morphologically studied and clarified the haemocytes immune response using Scanning Electron Microscopy (SEM). A marked significant increase was observed in the lysozyme activity of control with time. This may be due to the aging process of the larval instar. The lysozyme activity of the haemocytes showed significant increases at 6 and 12 h. However, the activity decreased significantly at 24 to 72 h, reaching nearly half that of the control value 72 h after the larvae were treated with farnesol. The presented results emphasize the importance of the insect haemocytes lysozyme activity which is considered as a safe measure for insect pest control.

Key words: *Bacillus thuringiensis*, farnesol, lysozyme, haemocyte response, SEM

INTRODUCTION

The black cutworm *Agrotis ipsilon* (Hufnagel), larvae, has a wide range of hosts, feeding on nearly all vegetables and many important grains and is a major agricultural pest in Egypt. The larvae feed readily on weeds, until weeds destruction, and then the larvae are enforced to feed exclusively on crop plants, aggravation damage. The larvae begin feeding on corn plants, however; they will feed aboveground until about the fourth instar. Once the fourth instar is attained, larvae can do considerable damage by severing young plants, in a single night. Corn is the most susceptible plant to black cutworm damage when it is less than 15 inches in height, although plants as tall as shoulder height are sometimes attacked by the larvae (Abdel-Gawaad and El-Shazli, 1971). The black cutworm controlled by disturbing their physiological activities and immune systems.

The *Bacillus thuringiensis*, entomopathogenic bacteria, are used to control numerous insect pests. The commercial formulation var. *kurstaki* Dipel 2X toxicity occurs after ingesting the crystalline spores which are soluble in the alkaline midgut, cause disruption of the insect gut epithelium, and are then proteolytically cleaved to release the active endotoxin to hemocoel (Broderick *et al*., 2006). Several investigators studied the immune response of circulating

haemocytes to *B. thuringiensis* in ixodidae tick (Inoue *et al*., 2001; Habeeb and Abou El-Hag, 2008). In addition, the free haemocytes of lepidopterous insects have been studied in a wide range of species (Andrade *et al*., 2003). Abd El-Aziz and Awad (2010a and b) illustrated the immune response and the activation of the humoral immune system that defends itself in A. *ipsilon* against infection with *B. thuringiensis* and diflubenzuron.

Plant essential oils produce secondary metabolites, terpenoids, which could be used as alternatives to conventional pesticides (Waliwitiya *et al*., 2005; Shaigan *et al*., 2008; Ebadollahi *et al*., 2010; Sujatha *et al*., 2010). These secondary metabolites are bioactive compounds, cause extent of disrupt the immature and adult emergence, antifeedants and toxicity in Lepidopteran pests. Sadek and Anderson (2007), Ahmed *et al*. (2009) and Cardenas *et al*. (2012) mentioned that the target pests are biodegradable the bioactive metabolites to non toxic products. Also the qualitative analysis of the plant essential oil revealed the presence of several chemical groups such as alkaloids, tannins, flavonoids, lignans and terpenes that might be responsible for the plant activity (Akomo *et al*., 2009). The purified farnesol, one of the plant terpenes, has been studied by Awad (2001) towards *A. ipsilon* larvae to show their insecticidal effects, as the terpenes has the action of the juvenile mimics hormone increase and impeded the molting process (Devanand and Rani, 2008; Silva *et al*., 2012).

Lysozymes are localized in various sites of the insect body. They are able to hydrolyze β-1-4-glucosidic linkages between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan of the bacterial cell wall. Moreover, lysozymes in the insect hemolymph are important in the insect immune defense system (Meshrif, 2007; Ursic-Bedoya *et al*., 2008). Lysozymes are also part of the defense mechanism against bacteria which has been described in insects (Dunn, 1986). The use of haemolymph as a medium for controlling insect pests has been made because the changes occurring in the haemolymph are quickly transferred to other portions of insect's body (Pugazhvendan and Soundararajan, 2009).

The aim of the present study was to evaluate the success of the microbial bacteria, and the non microbial growth regulator, farnesol, against the black cutworm larvae using the activity of lysozymes in larval haemolymph as an indicator. The present study also extended to examine the effects of *B. thuringiensis* and farnesol on the haemocytes immune response and their morphological changes using Scanning Electron Microscopy (SEM) to improve the strategies for using both of them in integrated pest management programs.

MATERIALS AND METHODS

Insect: Fourth instar larvae of *A. ipsilon* were used in the present study. The larvae were maintained at 25±3°C and 65±5% RH and a photoperiod of 12:12 (L:D) h. in the laboratory of the Entomology Department, Faculty of Science, Cairo University. They were fed on castor bean leaves (*Ricinus communis*).

Insecticides: Assay of the commercial formulation Dipel 2X, *B. thuringiensis* kurstaki was carried out. Six serial dilutions, 1000, 500, 250, 125, 62.5 and 31.25 μg g^{-1} of *B. thuringiensis* were prepared and applied in the present study. The assay procedure was performed according to Dulmage *et al*. (1970).

The tested pure terpene (3, 7, 11-trimethyl-2, 6-10-dodecatrien-1-01), sesquiterpenoid, farnesol (95%), was purchased from Sigma Chemical Co. A stock solution of farnesol was prepared. Five drops of Tween 60 as emulsifier were added to five drops of ethyl alcohol (95%), and then mixed

thoroughly with 1 mL farnesol, finally 100 mL of water was added to obtain a concentration of 1% (mL) farnesol emulsion. The lower concentrations were prepared by dilution of the above stock concentration with water. Six serial dilutions 1, 0.50, 0.25, 0.125, 0.63 and 0.30% (mL) of farnesol were examined.

Fresh castor bean leaf discs were sprayed by the use of atomizers with either 5 mL of *B. thuringiensis* or farnesol at each of the above mentioned concentrations. Then the leaf discs were left to dry and offered to 15 newly emerged fourth instar larvae placed separately in 15 cm diameter petri dishes for 48 h. Then the larval mortality was recorded. Leaf discs of castor bean leaves treated with water and one drop of tween 80 served as control. Five replicates were used for each concentration. Data on mortalities were calculated using Abbott (1925).

The LC_{30} was calculated and selected as a sub lethal concentration of bacteria and farnesol and used for further studies on the haemocytes.

Studies of haemocytes: Haemolymph was aseptically collected from larvae by cutting the last larval prolegs. Haemolymph was collected after 6, 12, 24, 48 and 72 h for control larvae and larvae treated with bacteria and farnesol at a concentration of LC_{30} for assay of the lysozyme activity of the haemocytes. The hemolymph was also collected after 48 h for SEM examination, in chilled sterile tubes. The modified method of Boucias *et al.* (1994) was adopted. The haemocytes were removed by centrifugation (Hettich MICRO RAPIDIK, Hettich Zentrifugen, D-7200 TutHingen, Germany) at 5000 rpm for 15 min. The supernatant, referred to as plasma, was transferred into a sterile Eppendorf tube and stored at 20°C until use.

Electron microscopy (SEM): The SEM method of Eisenback (1985) was applied. Haemolymph was diluted (1:1) with a cold physiological saline buffer containing 6% (v/v) glutaraldehyde and chilled for 30 min. The fixed cells were centrifuged at 6000 rpm for 2 min. The pellet was suspended in 0.1 M cacodylate buffer, pH 6.5, containing 2% osmium tetroxide and the mixture was incubated for 2 h at 4°C. The post-fixed cells were gradually dehydrated in a graded ethanol series (10% increments), submitted to CO_2 critical point drying, spattered with colloidal gold and studied by using JEOL JSM- 35C SEM at 15 kV.

Lysozyme activity of the haemocytes: The lysozyme activity was measured in the haemocytes of the 4th instar larvae of *A. ipsilon* using the enzymatic assay of lysozyme (EC 3.2.1.17) according to SIGMA instructions which are based on Shugar (1952).

Statistical analysis: The data obtained were statistically analyzed using one-way ANOVA followed by LSD post hoc for comparison between means of treated and non-treated groups. All calculations were performed using SPSS computer software program (version 14).

RESULTS AND DISCUSSION

Bioassay: The LC_{30}, LC_{50}, and LC_{90} of *B. thuringiensis* were 199.1, 278.2 and 320.2 μg g^{-1} and that of the farnesol were 0.089, 0.360, and 1.000% mL, respectively. The mortality percentage of 4th larval instar of *A. ipsilon* was increased by increasing the concentrations of *B. thuringiensis* and farnesol and also by increasing the post treatment time (Table 1).

Table 1: Insecticidal effect of *B. thuringiensis* and farnesol at different concentrations on the 4th instar, *A. ipsilon*, larvae

	Accumulative larval mortality at different post treatment times (%)					
	B. thuringiensis (μg g^{-1})			Farnesol % (mL)		
	24 h	48 h	72 h	24 h	48 h	72 h
LC_{30} = 199.1(μg g^{-1}) *B. thuringiensis* and 0.089% (mL) farnesol	0	28	29.5	12	30	38
LC_{50} = 278.2 (μg g^{-1}) *B. thuringiensis* and 0.360% (mL) farnesol	36.5	50.4	86.2	38	49	53
LC_{90} = 320.2 (μg g^{-1}) *B. thuringiensis* and 1.000% (mL) farnesol	54	88.6	100	78	88	100
Control	-	-	-	-	-	-
Slope	2.231	1.9	1.62	1.4×10^{-4}	1.6×10^{-4}	1.4×10^{-4}
Variance	759.08	938.89	1395.9	1105.3	874.3	1046.3

Studies of haemocytes: SEM studies of the haemocytes were carried out to elucidate the surface and external structure of the haemocytes from the 4th larval instar of *A. ipsilo*n treated with bacteria and farnesol. This study supports the information obtained by light and transmission electron microscopy observation from external morphological and functional view.

Five types of haemocytes were recognized in *A. ipsilon* larvae. They were previously characterized morphologically and classified according to Gupta (1979). Figure 1 shows the normal haemocytes of the 4th instar larvae of *A. ipsilon*. The prohaemocytes (Prs) are clearly distinguished by their surface structure, shape and size and are sometimes confused with the plasmatocytes (Pls). However, Pls are recognized as spindle-shaped, leaf-shaped, sickle-shaped, pear-shaped and round-shaped and the distinction between Prs and Pls is occasionally unclear, although the typical cells have characteristic profiles by SEM. The granulocytes (Grs) are covered with fibrous materials, nylon threads and many cytoplasm projections. Both Grs and Pls function in defense against foreign bodies. The spheriolucytes (Sps) are distinguished by their surface structure and are usually round and flat, with cytoplasm appearing lobulated and a characteristic surface pattern attributed to the presence of spheriole inside. The oenocytoids (Oes) are few large haemocytes detected in aggregated materials as giant cells, ovoid, rod-shaped, elongated, fusiform or even triramus according to the observation of Arnold (1982). Cells contact each other by their cytoplasmic processes. The healthy haemocytes have well developed cell membrane. The Pls cell membranes appear with acutely terminating thin pseudopodia radiating from the cell border. The surface of Prs is raised into somewhat concentric circles. Other cells resemble Prs in size but due to the presence of surface projections, they are similar to Grs entitling them to be called Pr-Gr intermediates, as it has been reported in some other insects (Pelc, 1986). Podocytes (Pos) have also been observed; they are flat cells with three or more cytoplasmic arms projecting out from a central body and also showing surface pores with variable number of arms which are greatly shortened in some cells. Due to their elongated spindle-shape, they seem to be derived by the elongation of the Pls. Our data on their relative percentage tend to support this view. Several authors also considered Pos as separate hemocyte types (Saxena *et al*., 1988; Jalali and Salahi, 2008; Pandey *et al*., 2010).

The 4th instar larvae of *A. ipsilon* treated with *B. thuringiensis* and farnesol revealed several pathological deteriorations (Fig. 2, 3).

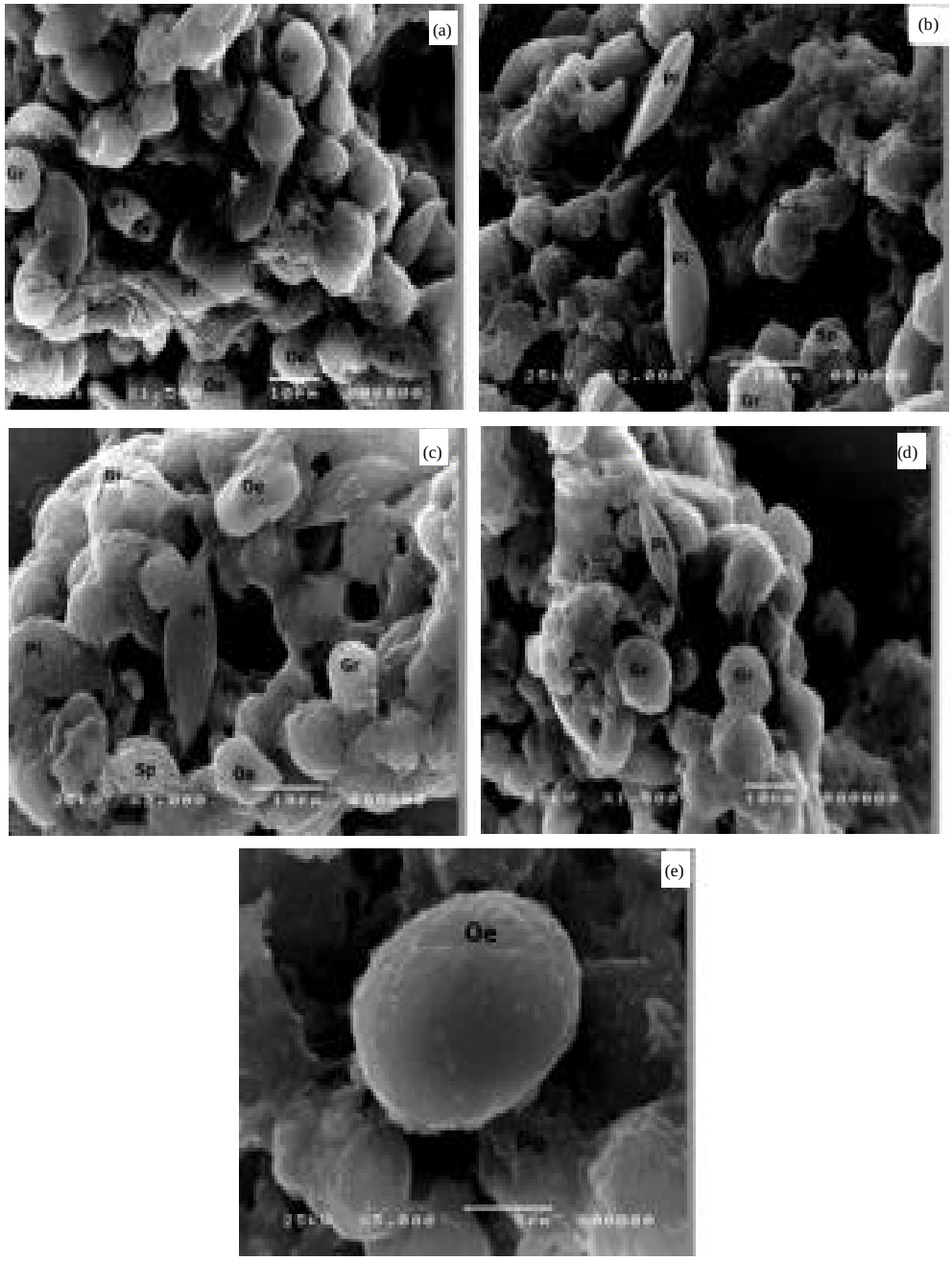

Fig. 1(a-e): Scanning electron micrograph (SEM) showing haemocytes from control larvae of the 4th instar *A. ipsilon*. (a, c and d) different population of haemocytes, cone-shaped Pl with a depressed bottom bearing pores (black stars), Pos possibly in different phases of activity, showing surface pores and variable number and sizes of arms, (b) Pr-Gr intermediate, (size of Pr and surface structures like Gr), (e) Oe-spherical and rough surfaced

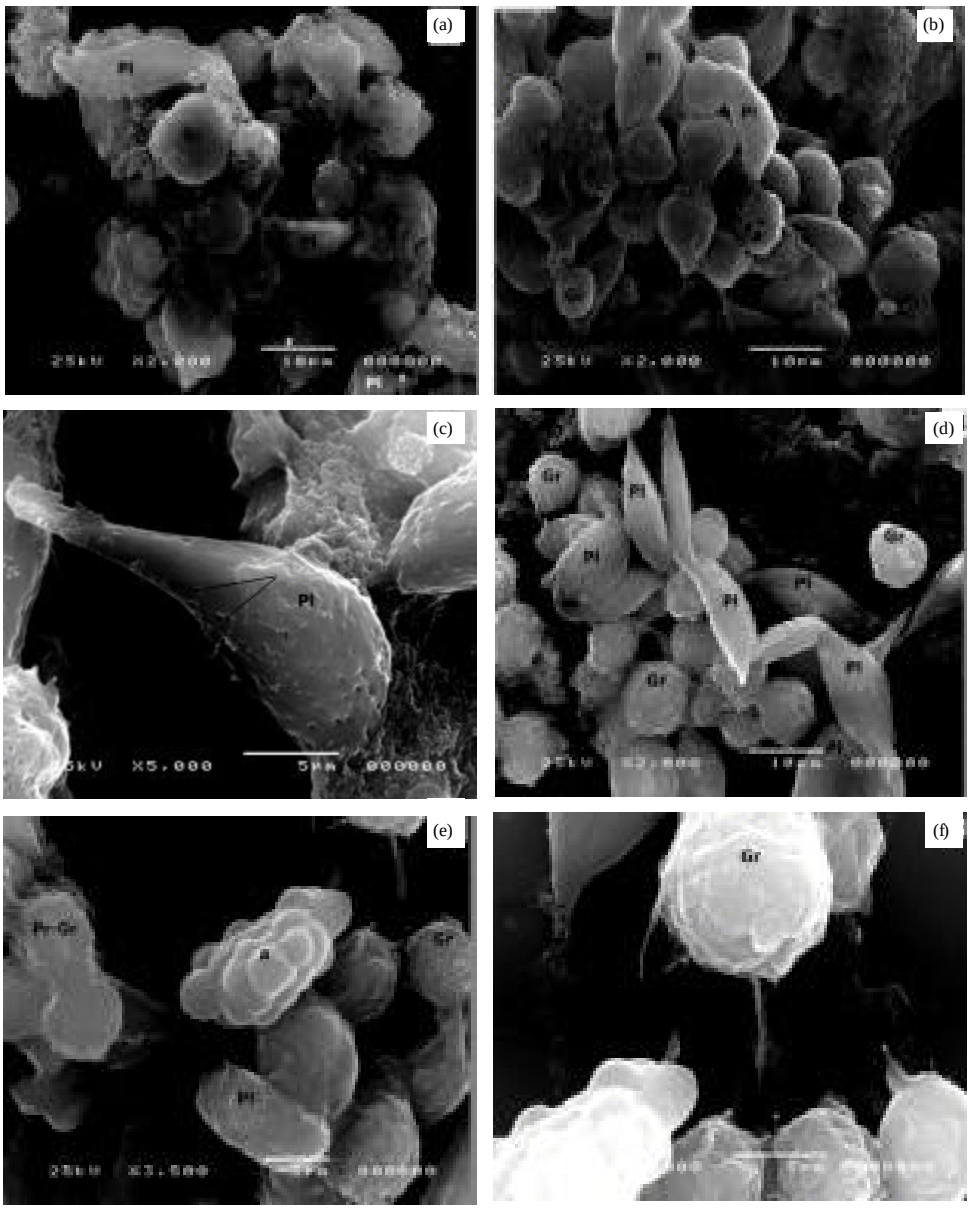

Fig. 2(a-f): Scanning electron micrograph (SEM) showing haemocytes from 4th larval instar *A. ipsilon*, treated with *B. thuringiensis*. (a, b, d and f), showing structural changes of the haemocytes which seemed swollen, deformed with irregular shrinkage of the cell membrane which seemed to be vacuolated. The surface of the cell is scattered with membrane folds and processes giving the cell a rough appearance, (e) Group of haemocytes in aggregations appeared to have intact cell membranes, (c) Pl with many vacuoles on the surface (line) Grs began to adhere to each other with pseudopodia, Pl with a gaping mouth on its surface at the periphery showing the process of phagocytosis of bacteria (arrow)

Figure 2, shows the haemocytes treated with *B. thuringiensis* with numerous structural changes; the plasma membrane of the Pls and Grs in Fig. 2 (a, b and d) show curled appearance, shrinkage, and the haemocytes became swollen giving the appearance of vacuolated cells, the vacuoles possibly enclosing the bacterial spores.

The outer surfaces of the Grs extend into long filaments due to the effect of bacterial toxins. The toxins secreted by bacteria enforce Pls and Grs to engulf the bacteria by the pseudopodia resulting in a clear rupture of parts of the cell membrane that have a very irregular outline bending at the cell boundary and this increases the phagocytic activity of the Pls and the Grs in Fig. 3(c and f). The phagocytosing haemocytes contain intracellular *B. thuringiensis* and attached bacteria may also enter the treated haemocytes, leading to the deformation and lyses of the cell membrane of

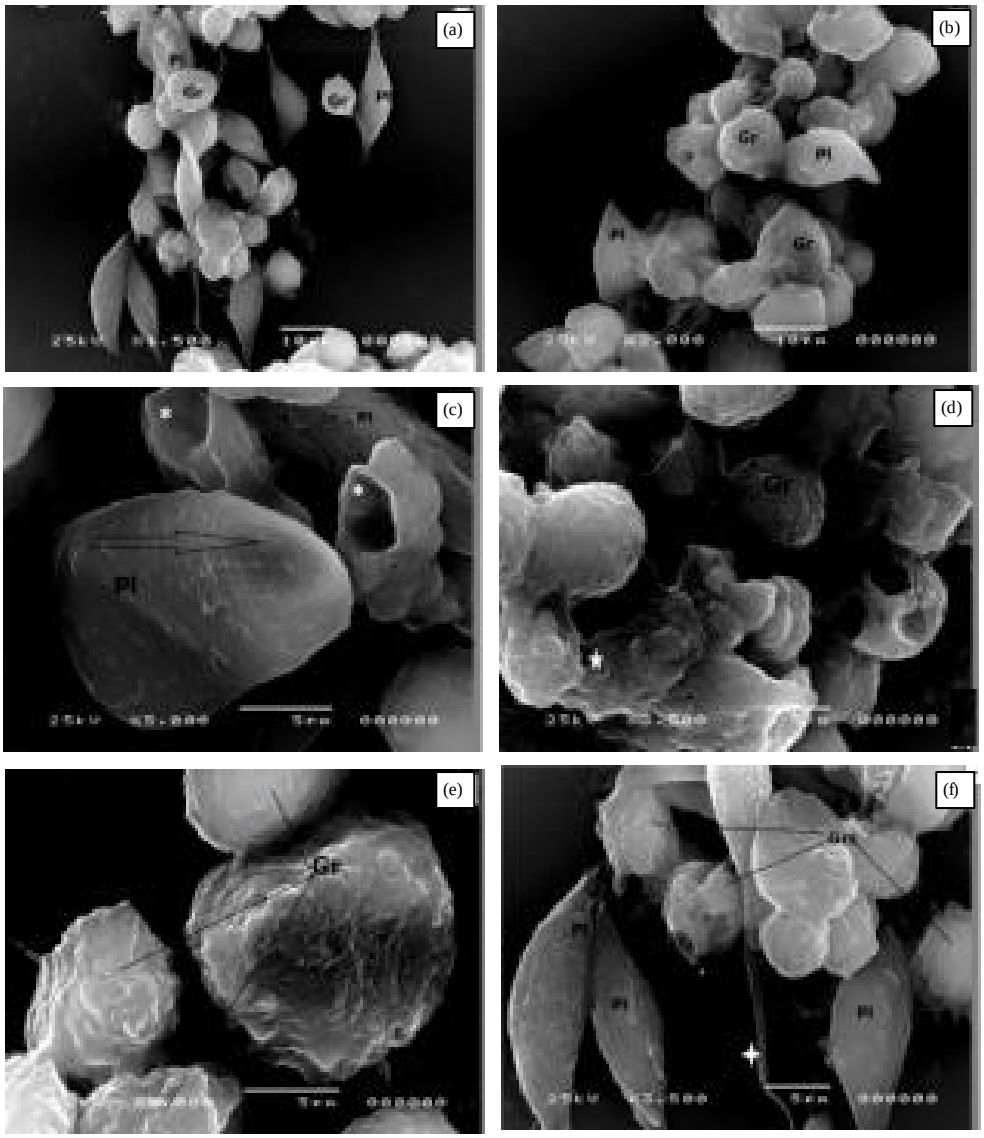

Fig. 3: Continue

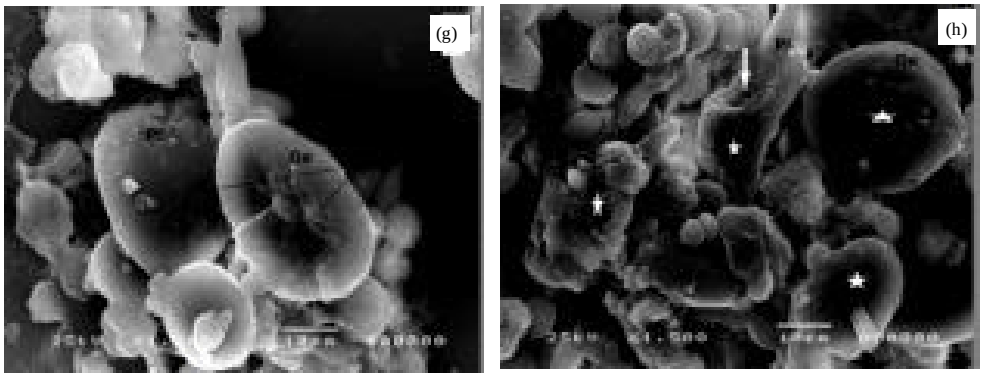

Fig. 3(a-h): Scanning electron micrograph (SEM) showing haemocytes from 4th larval instar *A. ipsilon*, treated with farnesol (a and b) Structural changes of the Grs and Pls, deformed with irregular shrinkage cell membrane, cells adhere to each other to form unstructured aggrigation. (c) Vacuolated Pls (line), cone shaped Pl with a depressed bottom bearing pores (white stars), Pl inamoeboid movement, irregular boundary and dark region (arrow), (d) Aggregated deformed, irregular shrinkage and rupture haemocytes (white star), vacuolated haemocyte (arrow), (e and f) Deformed Grs with irregular shrinkage cell membrane and the cells began to adhere to each other with pseudopodia (4-point star) to form unstructured aggregations which may later form nodules, (g and h): Aggregated deformed, irregular shrinkage Oes and rupture haemocytes with dark regions (stars). Pl with a gaping mouth on its surface at the periphery showing the process of phagocytosis (white arrow)

the treated haemocytes. These reactions represent a normal consequence because blood cells are the first to be invaded when the toxins reach the haemocoel. Similarly, Fuguet and Vey (2004) observed ultrastructural alterations in circulating plasmatocytes and granular haemocytes of *G. mellonella* larvae treated with several strains of *B. bassiana* and toxic doses of destruxins (cyclic peptide toxins). Haemocytotoxin lipopolysaccharide released from the bacteria resulted in the damage and death of the haemocytes (Dunphy and Webster, 1991; Mahar *et al.*, 2005). The destruction of the haemocytes may also be due to the margination effect, where the haemocytes tend to adhere to the wall of the haemocoel and to each other, after which some cells return into circulation (Chian and Anderson, 1983). Moreover, it has been demonstrated that the primary defence response was encapsulation; the haemocytes attach to the invaders forming small multilayer capsules from the adhesive and spreading haemocytes. The effect of terpenes from phytoextracts on haematological defense in the insects infected bacteria is not available. Phytoextracts, (having antimicrobial properties), on the haemocyte mediated response was reported by Kumar *et al.* (2012) and Pandey *et al.* (2012). Pech and Strand (1995) studied the encapsulation phenomenon in Lepidopteran, *G. mellonella*. They found that the Grs that come in contact with foreign particles undergo lysis or degranulation or release materials that promote the attachment of Pls to Grs. Also Broderick *et al.* (2006) illustrated that the bacterial spores, *B. thuringiensis*, from the infected mid gut epithelial cell lysis were released into the haemocoel where they germinated causing cytotoxic effects to the haemocytes.

Similarly, the haemocytes of larvae treated with farnesol showed clear shrinkage, swelling, darkening, malformation, vacuolation, irregular cell boundary and dark regions (Fig. 3). The cell surface showed membrane folds and surface processes were scattered giving the cell a rough appearance. A large number of surface pores in Pls, Grs, and Oes were also observed. The changes in the haemocyte response to insecticides after their siege could result from the harmful effects of their products that lead to the loss of the detoxifying role of the haemocytes. Similar findings were also reported by Andrade *et al.* (2003) in *Anticarsia gemmatalis*. Also, Essawy *et al.* (1985) in their SEM study noticed a large number of surface pores in the Pls of *Heliothis armigera*, whereas Saxena *et al.* (1988) reported only fewer of them in the cells of *Spodoptera litura*.

Figure 2 and 3 show that different groups of haemocytes in aggregations appeared to have intact cell membranes. These aggregations of the haemocytes were encapsulated, or the haemocytes themselves form nodules. The cellular aggregates were formed around bacterial cells or farnesol particles; adhesive flocculent material was released by Pls and Grs. Granule discharge occurred by localized breakdown of the cell membrane not by exocytosis. The late stages involved encapsulation and melanization (Ratcliffe and Gagen, 1977). Similar observations were also reported by Abd El-Aziz and Awad (2010a) who clarified the cytopathological alteration in the studied haemocytes and their immune response in *A. ipsilon* larvae infected treated with bacteria and dimilin using TEM. Pandey *et al.* (2010) also corroborate that the temperature regimes also affected the defense mechanism of *A. mylitta* larvae, an increase in number of haemocytes may be attributed to the detachment of hemocytes from tissue surfaces and the higher rate of hemocytes multiplication or production while the hemocytes clumping attributed to physiological response. Tojo *et al.* (2000) illustrated that in *G. mellonella*, the Pls were active phagocytes against large size particles. It can be hypothesized that the phagocytosis by Pls in *A. ipsilon* could be in fact a beginning of encapsulation process which is one of the functions of Pls in Lepidopteran insects. Ling and Yu (2006) have also shown that both microbial and non microbial particles were engulfed by Grs. Moreover, no can deny that the presence of secondary lysozymes, numerous pseudopods and acid phosphatase in vesicles from Golgi apparatus in Grs and Pls Associated with their function as phagocytes. The controversy on the phagocytosis process caused by lepidopteran Pls. Thus, the present study using SEM gives the best confirmatory results by revealing clearly the typical morphology (marginal spherule bulges) of these cells.

Lysozyme activity of the haemocytes: Table 2 shows that the hemolymph lysozyme activities of 4th instar *A. ipsilon* larvae increased significantly ($p \leq 0.05$) at 6 and 12 h, compared with the control insects. The larvae treated with bacteria recorded 310.75±26.41 μg mL^{-1} min^{-1} and 283.75±1.65, μg mL^{-1} min^{-1} and those treated with farnesol showed 409.25±5.53 and 334.75±1.84 μg mL^{-1} min^{-1} post treatment, respectively.

Table 2: Determination of the lysozymal activity (μg mL^{-1} min^{-1}) in the plasma of the 4th larval instar of *A. ipsilon* at different time intervals post infection with LC_{30} *B. thuringiensis* and farnesol

Time post infection (h)	Control (Mean±SE)	*B. thuringiensis* (Mean±SE)	Farnesol (Mean±SE)	Sig.
6	206.75±2.36^{a}	310.75±26.41^{b}	409.25±5.53^{c}	*
12	258.88±3.96^{a}	283.75±1.65^{b}	334.75±1.84^{c}	*
24	584.75±0.63^{a}	379.50±3.07^{b}	478.75±1.25^{c}	*
48	588.75±1.25^{a}	427.50±25.86^{b}	465.00±2.04^{b}	*
72	788.75±2.39^{a}	538.75±1.25^{b}	357.75±1.32^{c}	*

n: 5 replicates per test, *The different letters are significantly different at $p \leq 0.05$, according to the least significant different (LSD) test

However, a marked significant decrease was observed in the enzyme activity from the haemocytes treated with bacteria and farnesol after 24 to 72 h compared to the control value. The lysozymal activity showed a significant decrease ($p \leq 0.05$) to nearly 1/2 that of the control value after the larvae were treated with farnesol at 72 h. The reduction in activity of lysozymes in haemocytes may be an evidence for release of the enzymes from the granules, (degranulation), in Grs and the Pls which were swollen and also to phagocytose foreign particles. Their lysozymes compartment may also contribute to lysozymal degradation against biotic and abiotic particles. Zachary and Hoffmann (1984) illustrated that immunocytological techniques indicated that the lysozyme was synthesized and stored in the granular hemocyte in *Locusta migratoria*. Lysozymes are a powerful tool of the humoral defense in insects and are considered to be the other partner of phenoloxidases that enable us to estimate the disease resistance (Adamo, 2004). It has been reported that lysozymes are localized in haemocytes like granular haemocytes, spherules cells and oenocytoids and the enzyme was charged from the cells during lysis or contribution in coagulation (Lavine *et al*., 2005). Abd El-Aziz and Awad (2010b) studied the humoral defense reactions using non-self recognition phenoloxidases mechanisms and they found a clear depression of the humoral immune system of *A. ipsilon* by dimilin and *B. thuringiensis*. The authors found that phenoloxidase activity in the haemolymph was significantly suppressed and the haemolymph of both bacteria-infected and dimilin-treated larvae showed appearance of new immune proteins. Hence, the increase of the lysozyme activity may be attributed to the reported increase in the Pls% and the Grs% from a previous study on *A. ipsilon* larvae, using transmission electron microscopy, this clarifies the vigorous endocytic activity against foreign invaders (Abd El-Aziz and Awad, 2010a). This was an indication that activation of the humoral immune system defends itself against the infection. At 72 h post treatment, the significant decrease in lysozymes activity may be attributed to the microbial or non microbial toxins that have been already under the control of other arms in the immune system so there was no need to activate lysozymes. A marked significant increase was also observed in the control by 206.75±2.36, 258.88±3.96, 584.75±0.63, 588.75±1.25, and 788.75±2.39 $\mu g\ mL^{-1}\ min^{-1}$ after 6, 12, 24, 48 and 72 h, respectively. This may be due to the aging process since the larval instar may be approached their period ends with the increase in time. The enzyme activation mechanisms appeared to differ with the developmental and physiological stage (Thomas-Guyon *et al*., 2009). The decrease in the lysozyme activity plays a role in the recognition of exotic materials in the studied insects.

These immune defense lysozymes are distributed in the haemocytes and other tissues of different insect species (Liu *et al*., 2004). The exhausted haemocytes struggle between toxins, bacteria and farnesol, and haemocytes are unable to recognize the non- self molecule anymore.

Gotz and Boman (1985) reported that the lysozyme alone is not responsible for the killing of bacteria but other antimicrobial proteins, cecropins and attacin together with the lysozymes destroy the bacterial cell wall. In insect haemocytes the contents of lysozymes enzymatically attack bacteria by hydrolyzing their peptidoglycan cell walls. Haemocytic responses feature direct interactions between circulating haemocyte and foreign invaders; these typically take place immediately after treatments. The success of the immune response depends on the number and the types of hemocytes involved in these mechanisms (Andrade *et al*., 2003; Ribeiro and Brehelin, 2006).

CONCLUSION

The humoral reaction involves synthesis and release of several antibacterial, immuno-proteins. A better understanding of current knowledge of insect haematology and insect biochemical mechanisms and cellular defense mechanisms pave the way for the proper pest control management especially by using a biocontrol agent after understanding their strategy in bypassing

in the insect defense mechanisms against foreign invaders. It has been stressed for the proper management of the pests especially by using various biocontrol agents. Further work is required and some what expected as earlier in vivo studies on insect cellular defenses against insecticides.

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