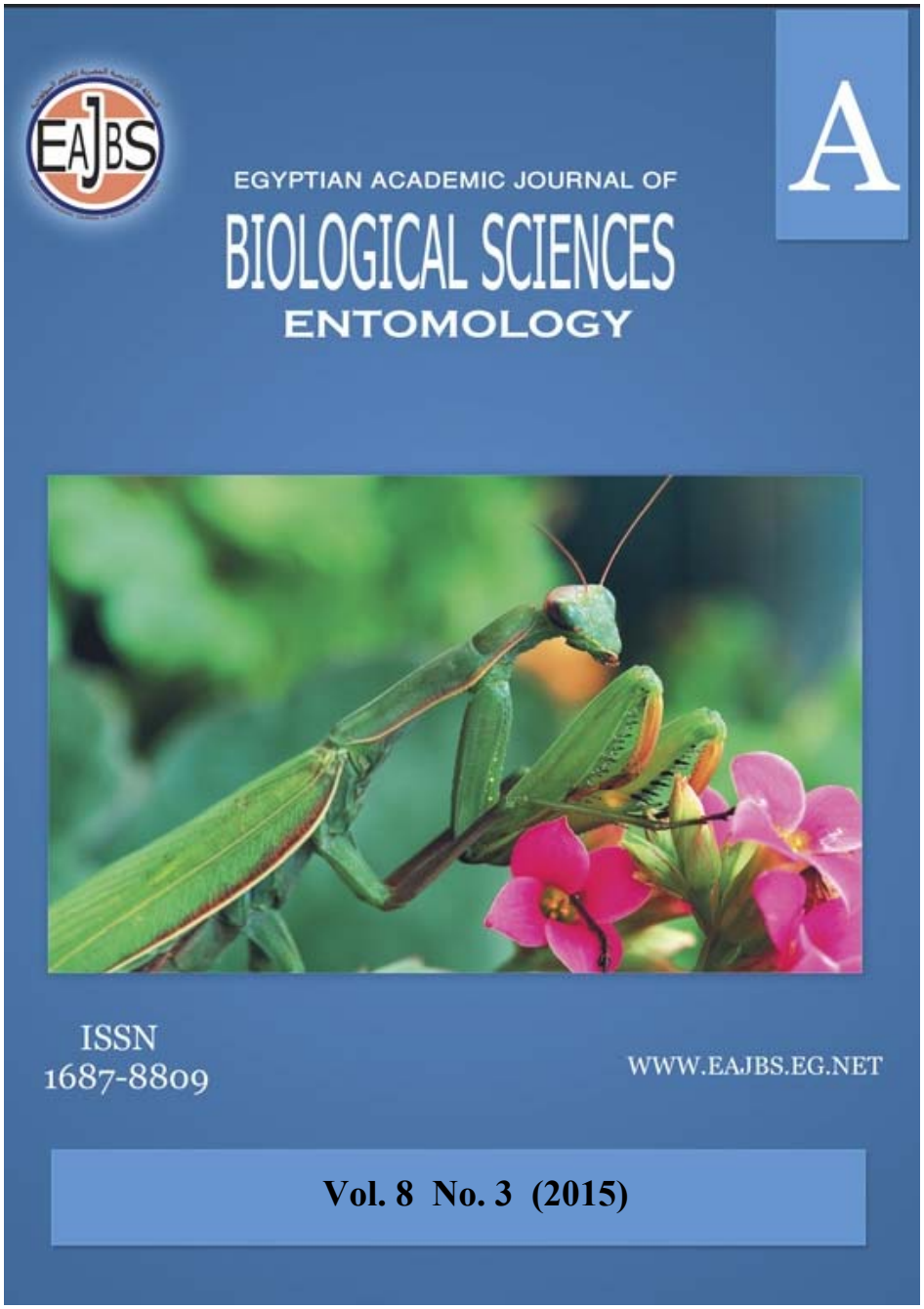

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Biochemical , Histological Studies of *Pectinophora gossypiella* (Saund.) Viral Infected Larvae Supported by Scanning Electron Microscope Inspections

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ABSTRACT

A lot of pink bollworm (PBW) *Pectinophora gossypiella* full grown larvae were excluded from cotton bolls collected from Sakha, Kafr El-Shiekh Governorate, Egypt. Larvae were transferred to laboratory of Bollworms Department, Plant Protection Research Institute, ARC, Dokki, Giza. The healthy appearance PBW larvae were placed individually in sterilized glass tubes (2x7cm) plugged with cotton wool under room conditions and observed 2 times/ week. Infected larvae with baculuviruses (NPV & GV) were collected. Present study clarified and discussed the pathogenicity of baculuviruses against PBW resting larvae in many views (appearance, histology and biochemistry). Results indicated that, the activation of PBW chitinase (CHT) at the resting period enhances the insecticidal activity of latent baculuviruses. So that the baculuviruses overcome apoptosis and prevent ecdysis of PBW resting larvae. And the scanning electron microscope and histology micrographs revealed the appearances of this infection as external scleroticzation, shrinking, swelling and internal degradation of *P. gossypiella* organs Furthermore, the biochemical analysis revealed decrease activity of larval chitinase with increase activity of both *N*-acetylglucosamine (NAG) and phenoloxidase. However, the utilizing of these epizootic baculuviruses as microbial control agents still need more studies of insect immune, insecticidal evaluation, molecular biology and environmental safety.

INTRODUCTION

Baculoviruses comprise a large family of arthropod specific viruses, the Baculoviridae, composed of four genera, the Alphabaculovirus (Lepidopteran-specific Nucleo polyhedron virus (NPV)), Betabaculovirus (Lepidopteran-Vspecific Granulovirus), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (Dipteran-specific NPV) (Jehle *et al.*, 2006 (c.f. Holly *et al.* 2010)). Ramanujam *et al.* (2014) reported that Baculoviruses is supposed to play an integral role in the natural regulation of insect populations but safety of the use of baculoviruses is still not clear.

Diapuased larvae of *Pectinophora gossypiella* collected from different localities in Egypt found to be inoculate naturally with latent viral infestation. These inoculated larvae exhibited the symptoms of viral infection at the end of resting period.

DNA probe testes indicated the presence of two baculoviruses, *P. gossypiella* GV (PgGV) and *P. gossypiella* NPV (PgNPV) and the DNA genome is a double-stranded DNA with molecular weight of 137 kb. The symptoms of PBW viral infection were recorded in details. Moreover, the percentages of successful emerged male and female adults from symptomatic PBW larvae were found to be (13.8% & 19.6%) and (17.5% & 24.9%), for the PBW populations of Assuit & Beni Souif, Governorates, respectively (El-Lebody 1998; El-Lebody & khtaab 2006; and El-Lebody *et al.* 2014).

Genetic and biochemical studies confirmed that baculoviruses have a gene encodes an ecdysteroid UDP-glucosyl transferase (egt). This enzyme catalyzes the transfer of glucose from UDP-glucose to ecdyste-roids, an insect molting hormone. Expression of the (egt) gene allowed the virus to interfere with normal insect development so that molting was blocked in infected larvae of fall armyworm, *Spodoptera frugiperda* (O'Reilly *et al.*, 1989). While Bhuvana *et al.* (1995) accepted the hypothesis that insect chitinase has potential to enhance the insecticidal activity of entomopathogens. On the other hand, two baculovirus genes, encoding a chitinase (chiA) and cathepsin (cath), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitinase is a chitin-degrading enzyme with endo- and exomol ecu lar specificity, whereas cathepsin has cysteine proteinase activity. (Slack *et al.*, 1995; Hawtin *et al.*, 1995). C.f. Valadez-Lira1a *et al.* (2011) stated that, Insects defend themselves against microbial pathogens by innate mechanisms, including increased phenoloxidase (PO) activity, but its relationship with microbial bio insecticides efficacy is little known.

The present work aimed to study some internal biochemical and histological effects of naturally viral infestation of *P. gossypiella* as well as the external inspection by a scanning electron microscope.

MATERIALS AND METHODS

Collection of *Pectinophora gossypiella* larvae:

More than 1000 blind cotton bolls were collected from cotton fields of Sakha, Kafr EL-Shiekh Governorate at the end of cotton season 2014. The collected cotton bolls were transferred to laboratory of Bollworms Department, Plant Protection Research Institute, ARC, Dokki, Giza. The PBW full grown larvae were excluded from cotton bolls. The healthy appearance PBW larvae were placed individually in sterilized glass tubes (2x7cm) plugged with cotton wool under room conditions and observed 2 times/ week.

Diagnosis of the viral disease larvae:

Through the experimental period the symptoms of viral infected PBW diapaused larvae were determined according to (El-Lebody & Khtaab 2006).

Biochemical studies:

1-Determenation of chitinase activity:

Substrate preparation and enzyme assay were carried out according to Bade and Stinson (1981) and Ishaaya and Casida (1974), respectively.

2-Determenation of N-acetylglucoseamine:

It was determined by the sensitive method of Waterhouse *et al.* (1961).

3-Phenoloxidase activity determination:

Phenoloxidase activity determined according to a modification of Ishaaya (1971).

Histological studies:

Microtomical sections of the 4th abdomen segment of both diseased and healthy larvae were done according to Gad (1951) and photographed.

Scanning electron microscopic inspection of viral infected PBW diapaused larvae:

The sympatric larvae as well as health ones were kept in glutaldhyde 4% for 24 hours; then washed with distilled water, dehydration in a series of alcohols and specimens were coated with a very thin layer (in nm) of gold–palladium using coater, and subjected to SEM inspection. Scanning electron microscope photo-graph of cuticle, head capsule, mouth parts, legs of (thorax, abdomen & anal) and anal plate of a healthy and an infected larva of *P. gossypiella* were obtained for demonstration and explanation of the viral effects against the PBW diapaused larvae.

RESULTS AND DISCUSSION

Biochemical effects of viral infection against *P. gossypiella*:

In this part of study three enzymes were subjected to analysis. Two of these enzymes are involved in insect moulting (chitinase and *N*-acetylgluc-osaminidases (NAG)) and the 3rd enzyme is more related to insect innate immune against the pathogens.

Biochemical analysis of chitinase and *N*-acetylglucosaminidases (NAG):

Insect chitinases and *N*-acetylglucosamine are among a group of proteins that insects use to ecdysis as well as they effect on chitin content. Both enzymes are found in the moulting fluid. The former enzyme hydrol -yzes chitin into oligosaccharides, whereas the latter, further degrades the oligomers to the monomer from the non-reducing end. (Vaaje-Kolstad *et al.*, 2005 C. F. Muthukrishnan 2012). Therefore, one of the potential functions of NAGs may be to prevent the accumulation of chitooligosaccharides at concentrations that are high enough to interfere with efficient degradation of chitin by CHT (Kramer and Muthukrishnan, 2005 C. F. Muthukrishnan 2012.

The present data in Table (1) indicate that both enzymes in infected larvae were influenced (14.2 ± 0.5 & 1238.6±55.4) compared to healthy one. (23.4±0.5 & 587±23.3) Ug NAGA / mim / g.bwt.

Table 1: Biochemical analysis of Chitinase and *N*-acetylglucosamine (NAG) and Phenoloxidase for viral infected healthy pink bollworm

Enzyme assays			
Chitinase (CHT)		***N*-acetylglucosamine (NAG)**	**Phenoloxidase (PO)**
UgNAGA/mim/g.bwt±SD		**UgNAGA/mim/g.bwt±SD**	**O. D. units/mim/g.bwt±SD**
Infected	**14.2 ±0.5**	**1238.6±55.4**	**18.8±2.3**
Healthy	**23.4±0.5**	**587±23.3**	**7.6±0.87**

The enzymes of moulting became active at the end of resting stage. The insect chitinase (CHT) has potential to enhance the insecticidal activity of latent virus. So that the expression of the virus (egt) gene allowed the virus to interfere with normal insect development then moulting was blocked and the insect chitinase (CHT) will be decreased. At the same time, the expression of the virus chitinase (*chi A*) cause dissolution of chitin of both the larval skeleton and gut PM. Moreover, the insect increase its *N*-acetylgl-ucosamine (NAG) to prevent the accumulation of chitooligosac charides (degraded chitin by virus chitinase (*chi A*). This result confirms the viral infection affects the PBW chitin metabolism and chitin content. In

this respect, precise regulation of chitin metabolism is a complex and intricate process that is critical for insect growth, metamorphosis, organogenesis, and survival (Arakane and Muthukrishnan, 2010 C. F. Muthukrishnan 2012). Also, the precise control of chitin content is critical not only for the survival of the insect, but also for optimal function of individual anatomical structures such as wings and other appendages. (Luschnig *et al.*, 2006; Wang *et al.*, 2006; Arakane *et al.*, 2009 C. F. Muthukrishnan 2012).

Biochemical analysis of phenoloxidase:

Phenoloxidase (PO) is involved in sclerotization of the cuticle and melanization associated with nodulation, encapsulation, and wound healing, and may provide cytotoxic quinonoid compounds to kill opportunistically invading microorganisms (Nappi and Christensen 2005 (C.F. Valadez-Lira1a *et al.* 2011).

The present result indicate that, viral infected *P. gossypiella* larvae had about 2.5 times more phenoloxidase activity (18.8 ±2.3) than healthy larvae (7.6 ± 0.87) O. D. units/mim/g. bwt. In this respect, Popham *et al.* (2004) C. F. Valadez-Lira1a *et al.* (2011) demonstrated that PO in the plasma of *H. virescens* might provide aconstitutive, humoral innate antiviral immune response to *Helicoverpa zea* single capsidnucleopol yhedrovirus (*Hz*SNPV) infection. Kent and Holly (2006) found that *Heliothis virescens* larval plasma contains high levels of an antiviral activity against the budded form of the *Helicoverpa zea* single nucleopolyhedrovirus (*Hz*S NPV). And add that In vitro the general inhibitors of melanization and specific inhibitors of phenoloxidase, completely blocked virucidal activity up to the level seen in controls. In addition they concluded that inhibitory compounds that diffuse from the lumen into the haemolymph may inhibit phenoloxidase activity in plasma, and may thereby alter susceptibility to viral infection.Valadez-Lira1a *et al.* (2011) stated that, insects defend themselves against microbial pathogens by innate mechanisms, including increased phenoloxidase (PO) activity. But, its relationship with microbial bioinsecticides efficacy is little known.

Histological Studies and Scanning Electron Microscope Inspections of *Pectinophora gossypiella* (Saund.) Viral Infected Larvae.

A- The inoculated *P. gossypiella* with viral infestation appeared normal in early and during diapause stage. In this regard, latent baculovirus virus sequences were only detected in the fat body of *M. brassicae* laboratory culture larvae (Hughes *et al*., 1997 C.F.ENV/JM/MONO (2002)). In addition, Rohrmann (2008) reported that, remarkable feature of NPV infection is that in some instances the insects can grow and continue feeding right up until they die. They appear healthy, yet when examined, are heavily infected with high concentrations of occlusion bodies in their cells and haemolymph. EGT (a gene of baculovirus) likely contributes to this effect. At the end of resting period, the symptoms of the latent virus infestation were observed either as dying of infected larvae; also deformations as larval/ pupal intermediate was noted. Moreover, dying and deformations of pupal and adult stages were recorded. The percentages of emerged moths were ranged between 14 to 25% according to their locality (El-Lebody *et al*. 2014).

In present study, histological and scanning electron micrograph of healthy larvae was placed on the left of plates for comparison with that of infected larvae on right of plates.

Scanning electron inspection of viral infected PBW larvae revealed the following points:

The baculoviruses infectetion blocking molting of *P. gossypiella*:

As shown in plate (1), the external inspection by a scanning electron

microscope reveal the exclusively presence of old cuticle of viral infested larvae adhering to and mask new integument at head capsule and anal plate (Figs.1&2, respectively). Also, Fig. (3) showed the old cuticle around an abdomen leg masked new integument (noticeable the sensitive hair of new integument at the left of figure). For this reason the larvae failed to moult. In addition, Fig. (4) show an increase in the integument thickness where it measured 36.52um compared to (7.5um) of healthy one (Figs. 4 & 5). Firstly, Kubo *et al.* (1981), studied insect ecdysis inhibitors from the East African medicinal plant *Ajuga remota* (Labiatae) *against P. gossypiella* and other pests, They found that, when phytoecdysones, cyasterone and ecdysterone (ecdysis inhibitors) fed to *P. gossypiella* ,apparently upset the temporal patterning of the moulting cycle because hardening (scleroticzation) of the newly synthesized cuticle occurred before its expansion, so that the previous cuticle remained adhering to, and thereby masking, the new cuticle. This process repeated itself until individual larvae were with up to three cuticular head capsules, all of approximately equal size and all adhering to one another. Secondly, blocking moulting of insect by the viral infestation may occur according to one or more of following reason (s).

1-O'Reilly *et al.* (1989) confirmed that, the insect baculovirus Autographa California nuclear polyhedrosis virus has get gene.

This gene encodes an ecdysteroid UDP-glucosyl transferase (egt). This catalyzes the transfer of glucose from UDP-glucose to ecdyst -eroids, which are insect molting hormones. Expression of the (egt) gene allowed the virus to interfere with normal insect development so that molting was blocked in viral infected larvae of *Spodoptera frugiperda*.

2- Bhuvana *et al.* (1995) accepted the hypothesis that insect chitinase has potential to enhance the insecticidal activity of entomopathogens.

3- Narayanan (2004) reported that, dissolution of chitin by chitinase (either from insects themselves or from fungi) is known to perforate peritrophic matrix and exoskeleton and make insects vulnerable to attack by different pathogens.

The baculoviruses infection caused and external scleroticzation, shrinking, swelling and internal degradation of *P. gossypiella* organs:

As shown in plate (2), generally, the baculoviruses infested PBW caused external scleroticzation and swelling of all organs, (head capsule (Fig.1), thorax legs (Figs.12-15), abdomen, abdomen leg (Figs.16-19) and anal legs (Figs. 21-22) except the thorax where it measured 1.99 um (length) & 1.89 um (width). The corresponding measurements of the healthy larvae were 2.3 um and 2.11 um, respectively. However, scleroticzation was discussed in the point A. Concerning the internal breaking down of organs, the author in previous studies recorded a ventral hole between the thorax and some of first abdomen segments. In present study, the micrograph revealed flaccidity as a result of breaking down of internal tissue (Fig. 14). In respect of abdomen legs, (Figs. 18 &19) show degradation of planta so that the crochets full down in the cavity of internally breaking down of abdomen legs. Also, the crochets of anal prologs are adhered together while in healthy case they arranged in a curved row on the anterior margin of the planta pedis.

However, the final step of baculovirus infection is the breakdown of the larval cuticle and the release of the occlusion bodies into the environment. Two baculovirus genes, encoding a chitinase (*chiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval carcass and the release of occlusion bodies. Chitinase is a chitin-degrading enzyme with endo-and exo-molecular specificity, whereas cathepsin has cysteine proteinase activity (Slack *et al*., 1995; Hawtin *et al*., 1995 (C. f ENV/JM/MONO (2002)1).

The baculoviruses infestation caused swelling of fat bodies of *P. gossypiella:*

Across section of the fourth abdomen segment, show swelling in fat bodies of moribund PBW larvae as a result of viral infection (Fig. 24).

This part of present study indicates two conclusions:

1-The latent viral infestation of *P. gossypiella* may due to viruses' evolved mechanisms that block the insect apoptotic response and so establish an infection; where baculoviruses have two classes of proteins, the p35 protein and the IAP which have been identified and characterized as anti-apoptotic agents (Clem *et al*. l991; and Clem, 1997 C.F.ENV/JM/MONO (2002)). Another mechanism is that, the baculoviruses (egt) gene may contribute to overcome apoptotic response; where a common method to reduce the time that a virus takes to kill its host is to delete the (egt) gene (Rohrmann 2008).

2- The insecticidal effects of viral infestation against PBW may due to tow baculovirus genes, encoding a chitinase (*chiA*) and cathepsin (*cath*), have been described to contribute to the liquefaction of the larval skeleton. (Slack *et al*., 1995; Hawtin *et al*., 1995 C.F.ENV/JM/MONO (2002). Also, dissolution of chitin by chitinase either from insects themselves or a pathogen (fungi) is known to perforate peritrophic matrix and exoskeleton and make insects vulnerable to attack by different pathogens (Narayanan, 2004).

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Plate 1

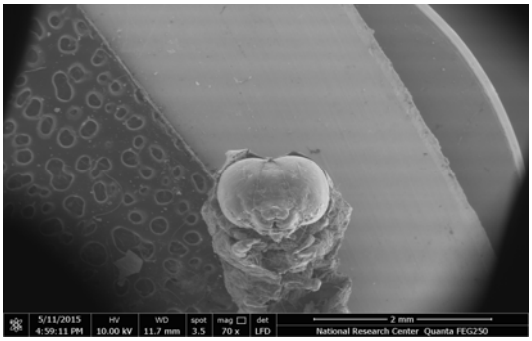

Fig. 1: PBW infected larvae can't remove premier cuticle, sclerotization & swelling of head capsule.

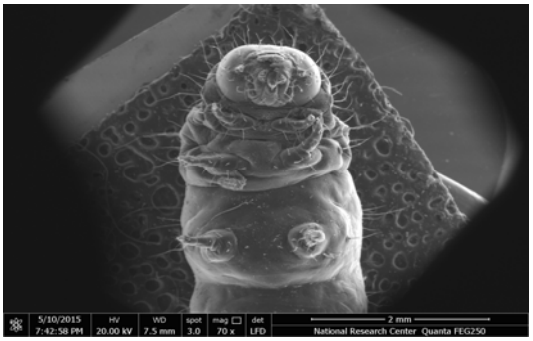

Fig. 2: PBW healthy larvae normal cuticle and head capsule.

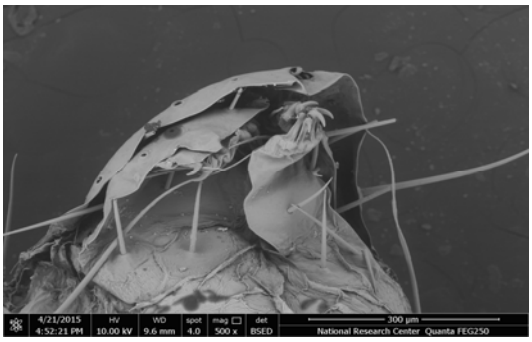

Fig. 3: PBW premier cuticle masked new one and adhere to anal plate.

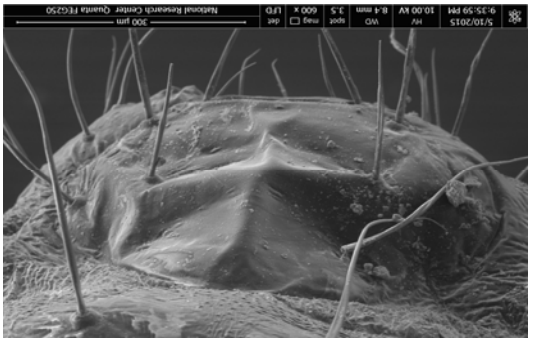

Fig. 4: Anal plate of PBW heathly larvae.

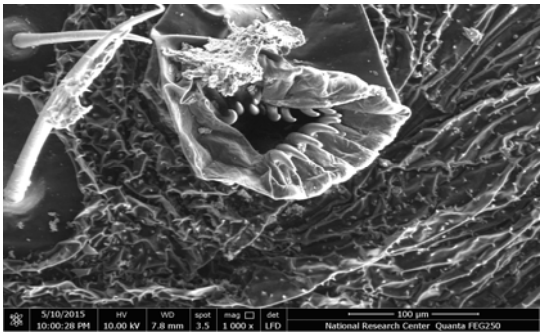

Fig. 5: PBW infected larvae premier cuticle masked new one at left of Fig.. noticeable sense hair and adhere to abdomen.

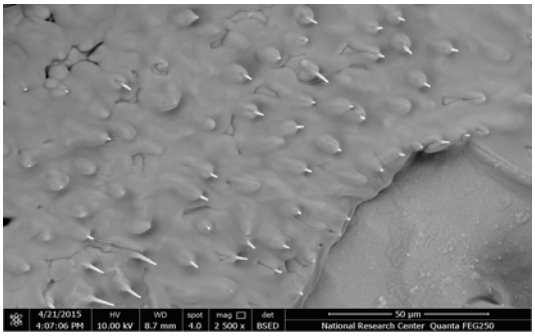

Fig. 6: Cuticle sclerotization of infected PBW larvae.

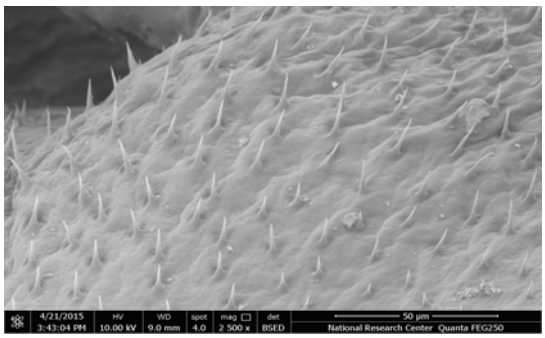

Fig. 7: Cuticle of heathlyPBW larvae.

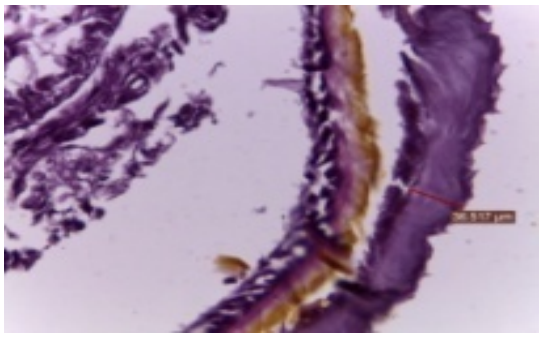

Fig. 8: Cuticle section of infested PBW larvae.

Plate 2

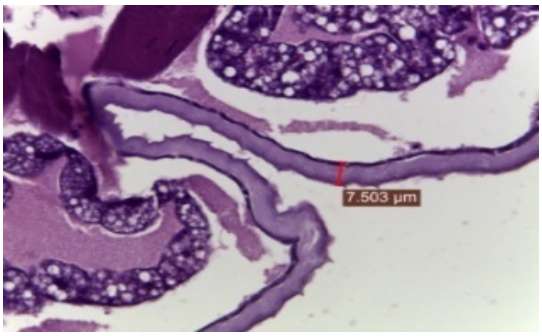

Fig. 9: Cuticle section of healthy PBW larvae.

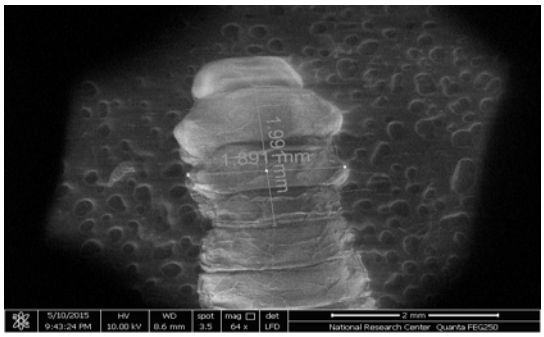

Fig. 10: Sclerotization and shrinking thorax of infested PBW larvae.

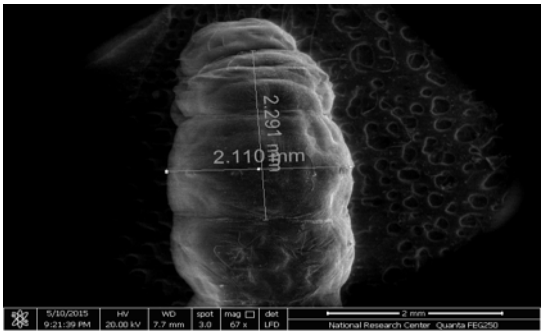

Fig. 11: Thorax of healthy PBW larvae.

Fig. 12: Thoracic leg sclerotization and shrinking of infected PBW larvae.

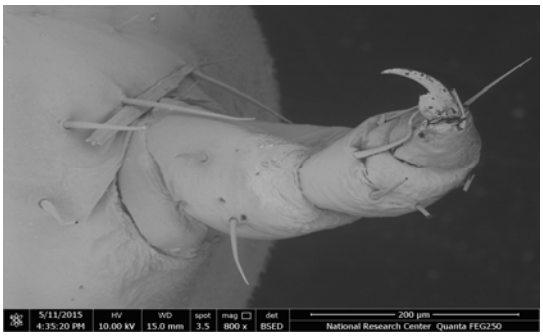

Fig. 13: Thoracic leg of PBW healthy larvae.

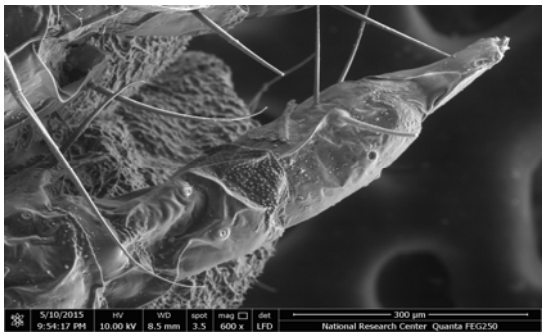

Fig. 14: Thoracic leg of PBW infected larvae with breaking down of internal tissue.

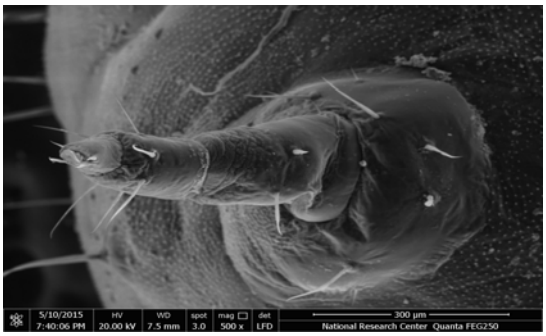

Fig. 15: PBW healthy larvae thoracic leg.

Fig. 16: Abdomen swelling and sclerotization of PBW infected larvae.

Plate 3

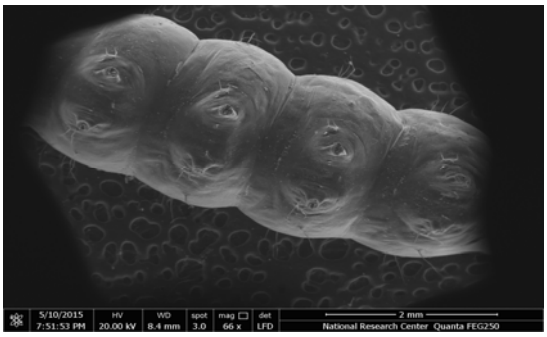

Fig. 17: Abdomen of healthy PBW larva.

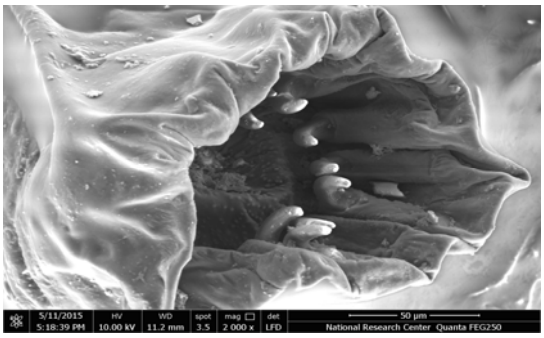

Fig. 18: Abdomen leg of PBW infected larva with degradation of planta pedis and chrochtes failed down into the body cavity.

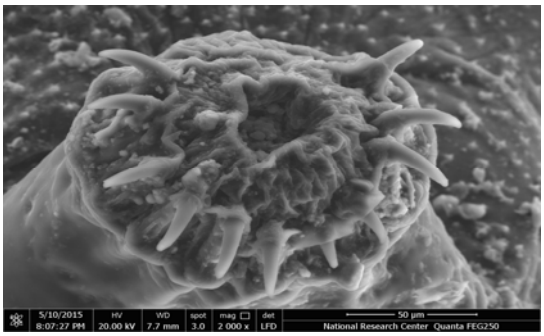

Fig. 19: Abdomen leg of PBW healthy larva with normal planta pedis and chrochtes.

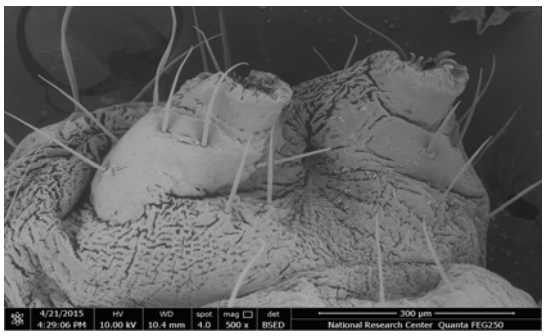

Fig. 20: PBW healthy larval anal legs (compare with Fig. (3)).

Fig. 21: PBW healthy larvae anal leg with degradation of planta pedis and chrochtes adhere together.

Fig. 22: PBW healthy larvae anal leg with normal planta pedis and chrochtes.

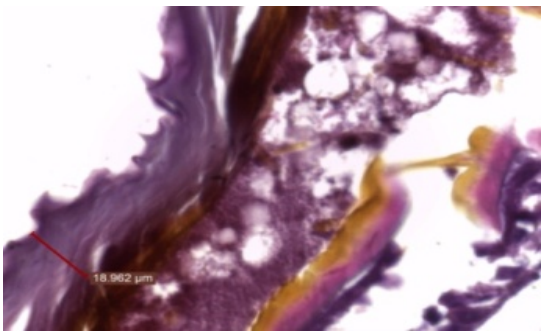

Fig. 23: PBW infected larvae fat bodies swelling and degradation.

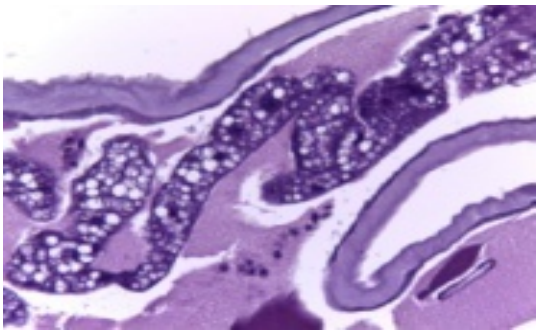

Fig. 24: PBW healthy larvae fat bodies swelling and degradation.

ARABIC SUMMERY

دراسات بيوكيميائية و هستولوجية مدعمة بالفحص بالميكروسكوب الإلكترونى ليرقات دودة اللوز القرنفلية المصابة بفيروسات Baculuviruses

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تم جمع حوالى 100ألف لوزة من القطن العالق من حقول القطن فى سخا بمحافظة كفر الشيخ فى نهاية موسم القطن 2014 للحصول على يرقات دودة اللوز القرنفلية الساكنة. تم حفظ اليرقات فى أنابيب زجاجية معقمة تحت الظروف الجوية الطبيعية للمعمل ومتابعتها مرتين / أسبوع. أجريت الدراسة على اليرقات التى أظهرت أعراض الإصابة بالفيرس. حيث أخذت بعض اليرقات للفحص بالميكروسكوب الإلكترونى وأخرى لدراسة الهستولوجى و ثالثة لقياس نشاط chitinase, *N*-acetylglucosamine (NAG) و phenoloxidase أوضحت النتائج أن إصابة فيروسات Baculuviruses لدودة اللوز القرنفلية تمنع الحشرة من التخلص من الخلايا المصابة (apoptosis) دون أن تظهر اليرقة أى أعراض للإصابة حتى نهاية فترة سكونها حيث تنشط أنزيمات السكون فى الحشرة ومنها إنزيم chitinase فيحدث نشاط للفيرس ويمنع إنسلاخ اليرقة إلى طور العذراء و تتمثل أعراض الإصابة فى إنخفاض نشاط إنزيم chitinase وزيادة نشاط إنزيمى phenoloxidase و N-acetylglucosamine (NAG) وعدم قدرة اليرقة على التخلص من جلد الإنسلاخ وتتصلب الحشرة خارجيا وتتحلل الأنسجة الداخلية مما يؤدى إلى موت يرقة دودة اللوز القرنفلية.