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Biocontrol Potential of Entomopathogenic Fungus, *Trichoderma Hamatum* against the Cotton Aphid, *Aphis Gossypii*

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Abstract: *Toxicological, biochemical, histological and ultrastructural studies of the impact of the entomopathogenic fungus, Trichoderma hamatum spore suspension on the adult cotton aphid (Aphis gossypii) were investigated in this study. The toxicological bioassay showed that the LC_{50} and LC_{90} against the adult aphid were 35.778 and 361.799 spores/ml of T. hamatum, respectively after 5 days post-treatment and the mortality percentages were 20.88; 26.39; 31.28 and 65.15% at 10^2; 10^3; 10^4 and 10^8spores/ml of T. hamatum, repectively. The biochemical analysis of the treated adult cotton aphid revealed different quantitative changes in the total soluble protein; transaminase enzymes and the carbohydrates hydrolyzing enzymes relative activities as compared to the untreated ones. Also, the histological and ultrastructural studies showed many alterations and malformation in the treated adult A. gossypii body and tissue, in addition to the development and the colonization of T. hamatum inside the insect tissues. Based on these results, T. hamatum could be suggested as a suitable biocontrol agent against A. gossypii.*

Keywords*: Aphis gossypii, biocontrol, cotton aphid, histological, Trichoderma hamatum, ultrastructural studies.*

I. Introduction

It is obviously that, insect pests are limiting factors for healthy growth of cultivated plants. Among those, aphids are one of the most important arthropod pests of greenhouse and field crops throughout the world [1]. Aphids are major insect pests of cereal crops around the world. For example, the cotton aphid (*Aphis gossypii)* is one of the key pests of cotton plants, where it causes serious quantity and quality damage and losses in yield [2]. Environmentally, microorganisms particularly fungi offer friendly alternatives to chemical pesticides that are give the pests developed resistance to conventional pesticides. Therefore there is considerable interest in the exploitation of naturally occurring entomopathogenic fungi for control of different insects including aphids and their diseases [3,4].

Fungi are unique compared to other microorganisms causing diseases in insect because they infect through the insect cuticle and do not require to be ingested, showing a great potential for controlling sucking insect such as aphids [5,6,7,8]. Fungi considered as important aphid biocontrol and the development of entomopathogenic fungi as aphids biocontrol agents has received increasing interest as important part of integrated control strategies [2,9,10,11].

Fungal diseases in insects are common and widespread. There are more than 700 species of entomopathogenic fungi currently known [12,13]**.** Entomopathogenic fungi infect their hosts through the cuticle, penetrate them and spread through the body and after the fungus has killed the host, it can grow out of the host cadaver and produce more spores, increasing the chance for others to be infested [14,15].

Trichoderma spp. has been widely used as antagonistic fungal agents against several pests as well as plant growth enhancers. Mycoparasitism, spatial and nutrient competition, antibiosis by enzymes and secondary metabolites, and induction of plant defense system are typical biocontrol actions of these fungi [16].

The present investigation aims to evaluate the biocontrol potential of *T. hamatum* againt *A. gossypii* and investigate the interaction between them as a pathogen and a host from some biochemical; histological and ultrastructural studies under laboratory condition.

II. Materials And Methods

II.1 The insect: The cotton aphids (*Aphis gossypii*), were collected from different fields in Sharkia, Esmalia and Port Said governorates, Egypt, put in paper bags and transferred to laboratory (Insect Pathogenic Production unit) at Plant Protection Institute in Sharkia, Egypt. The insects were individually reared in the laboratory at 25ºC±2 and 70%±10 relative humidity with 12 hours of photoperiod [17].

II.2 The microorganism: *Trichoderma hamatum* was isolated from cotton aphids by the homogenization technique followed by dilution plating of the homogenate on modified Czapeck's Dox's agar medium [18] in Plant Protection Institute in Sharkia governorate, Egypt. Cultures were maintained on PDA agar slants at 4ºC

and subcultured every 15 days. Then it was identified according to Domsch *et al.* [19]; Bissett [20] and Moubasher[21].

II.3 Biocontrol treatments:

II.3.1 Preparation of inoculum: Serial dilutions of spore suspension were prepared from *T. hamatum*. The spores were harvested by rubbing the surface of 7 days old plate culture of the tested fungus using sterile distilled water containing a drop of Tween 80 [22].The suspensions were filtered through cheesecloth to reduce mycelium clumping. The spores were counted in the suspensions using a haemocytometer to be 10^8; 10^4; 10^3 and 10^2 spores/ml.

II.3.2 Inoculation: The following leaf dipping technique was used as described by Krutmuang and Mekchay[22] and Ghatwary[23]. Fifty aphid mothers were counted and put in sterile Petri dishes, five dishes (n=5) for each treatment as replicates as well as control. The discs of cotton leaves (2 square inch) were prepared, dipped in the tested spore suspensions for 10 second, then left to dry at room temperature and provided to the aphid in Petri dishes. Controls were prepared in a similar manner using sterile distilled water. Mortality was recorded after 24, 48, 72, 96 and 120 hours post-treatments sub laboratory conditions (25ºC±2 and 70%±10 R.H.) using the Abbott's formula [24] as following:

$$\text{Mortality (\%)} = [(X - Y)/X]100$$

Where X = % survival in control,

and Y = % survival in treated aphids.

The concentration mortality regression analysis was computed for the tested fungus according to Finney[25] , for determining the fifty percent and ninety percent lethal concentrations (LC_{50} and LC_{90}, respectively).

II.4 Biochemical studies:

II.4.1 Preparation of aphid samples for the biochemical assay: The aphid samples in 1gm weight were collected from the untreated (control) and treated (10^8 spore/ml) aphids groups after 2; 3 and 4 days post-treatment and put in small bottles and kept in freezer until analysis.

The freezed samples of aphids were homogenized in distilled water 5ml/ sample, using Teflon homogenizer. The homogenates were centrifuged at 5000 r.p.m for 20 minutes at 5ºC. The supernatants were immediately used for the following chemical assay.

II.4.2 Determination of total soluble protein: Colorimetric determination of total soluble protein in supernatants of homogenate *A. gossypii* were carried out as described by Gornall, *et al.*[26].

II.4.3 Determination of transaminase enzymes activities (GPT & GOT): Glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) enzyme activities were determined colorimetrically according to the method of Reitman and Frankle[27].

II.4.4 Determination of carbohydrate hydrolyzing enzymes activity: The method used to determine the digestion of trehalose, starch and sucrose by trehalase, amaylase and invertase enzymes respectively, were similar to those described by Ishaaya and Swiriski[28]

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II.5 Light and electron microscopy: Groups of cotton aphids *A. gossypii* were infected with spore suspension (10^8 spores/ml) of *T. hamatum* and other groups were sprayed with water as control then incubated from 1-7 days at 25ºC±2 and 70%±10 R.H. and subsequently prepared for light and electron microscopy.

Slice tissue samples into ~ 1 mm slices. Slice tissue was processed for TEM by fixation in glutaraldehyde and osmium tetroxide, dehydrated in alcohol and embedded in an epoxy resin. Microtome sections prepared at approximately 500-1000 µm thickness with a Leica Ultracut UCT ultramicrotome. Thin sections were stained with cotton blue then sections were examined by the light microscope and photographed by camera Lica ICC50 HD. Then ultra thin sections prepared at approximately 75-90 µm thickness and were stained with uranyl acetate and lead citrate, then examined by transmission electron microscope JEOL (JEM-1400 TEM) at the suitable magnification. Images Were captured by CCD camera model AMT, optronics camera with 1632 x 1632 pixel formate as side mount configuration. This camera uses a 1394 fire wire boared for acquision [29,30]. (The work was done in TEM Lab. FA-CURP, FAC. Of Agric., Cairo University, Research Park).

III. Results And Discussion

III.1 Biocontrol test: Fungi have been proposed as biocontrol agents against a wide range of insects particularly aphids and evaluated according to their virulance [8,11,31,32]. The biocontrol test was conducted to determine

the LC_{50} and LC_{90} of *T. hamatum* spore suspension against adult *A. gossypii*. Fig. (1), shows the latent effect of *T. hamatum* spore suspension on the aphid adults after inoculation with different *T. hamatum* spores (10^2; 10^3; 10^4 and 10^8spores/ml). The mortality percentages were detected (calculated by Abbott's formula) after 1; 2; 3; 4 and 5 days post-treatments. It was found that the mean adult mortality percentages were increased with the increasing of spores concentrations and mortality percentages after 5 days post-treatment were 20.88; 26.39; 31.28 and 65.15% respectively. A linear relationship between the tested spores concentrations and the mean mortality percentages after 5 days post-treatment (Fig.2) was made by Probits analyses to determine the LC_{50} and LC_{90}. The obtained results revealed that, the LC_{50} and LC_{90} were 35.778 and 361.799 spores/ml, respectively after 5 days post-treatment. The obtained results appeared that the *T. hamatum* induced highly mortality against cotton aphids and this agree with Verma *et al*.[16] who reported that, *Trichoderma* spp. have been widely used as antagonistic fungal agents against several pests. Sahebani and Hadavi[33] showed that different concentrations of *T. harzianum* (10^2-10^8 spore/ml) decreased nematode infection significantly compared to control. Also, *T. album* caused 100% mortality within 5 days of inoculation against poultry red mites [34]. Van *et al*.[11] showed that twelve strains of different entomopathogenic fungi were screened for aphid control, among tested entomopathogenic fungi *Metarhizium anisopliae* and *B. bassiana* showed the highest virulent pathogenicity for both *Myzus persicae* and *A. gossypii* and their control values were both nearly 100% at 5 and 2 day after treatment, respectively.

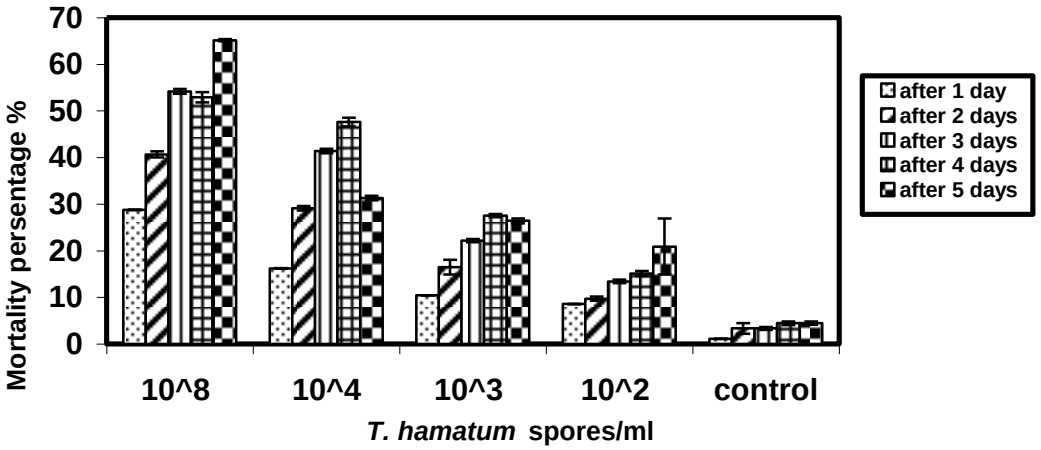


Fig. (1): Mortality percentages of treated adult *A. gossypii* with different *T. hamatum* spores concentrations (10^2; 10^3; 10^4 and 10^8spores/ml) after time intervals (1; 2; 3; 4 and 5 days) as compared to the untreated one (control).

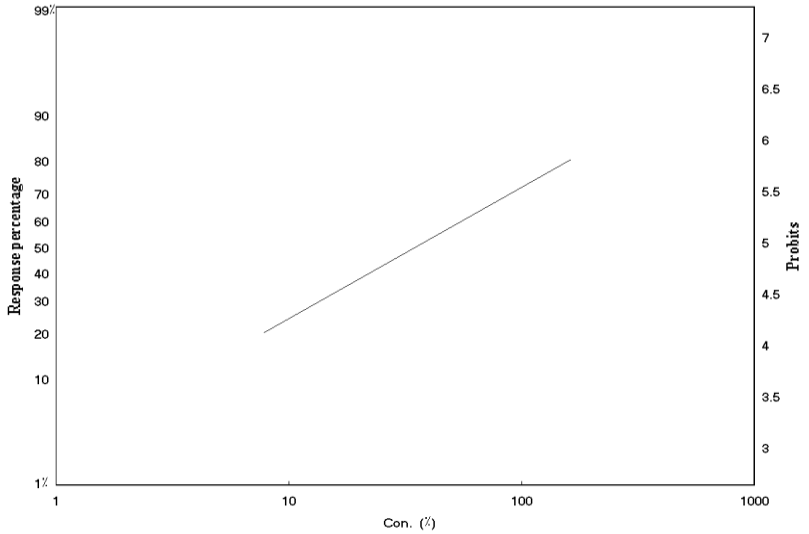


Figure (2): Concentration mortality probit line of *T. hamatum* spores/mL on adult *A. gossypii* after 5 days post-treatment to determine the LC_{50} and LC_{90}.

III.2 Biochemical analysis: The biochemical responses of adults *A. gossypii* expressed as total soluble protein; transaminase ennzymes (glutamic pyruvic transaminase GPT, glutamic oxaloacetic transaminase GOT) and the carbohydrates hydrolyzing enzymes (amylase, trehalase and invertase), using *T. hamatum* spore suspension

(10^8spores/ml). These attributes were measured at time intervals of 48; 72 and 96hrs. post-treatment. As appeared in Fig. (3), there is a disturbance in the relative activities in the assayed attributes as compared to that of control. Where, there is an increased in the total soluble protein by 34.27 and 29.18% after 48 and 72hrs, respectively while it decreased in 4.87% after 96hrs as compared to control. Protein is among the most important compounds of insect that bind with foreign compounds [35]. The results obtained by Padmasheela and Krishnan[36], showed an increase in total protein content in 3^{rd} instars larvae of scarabaeid *Oryctes rhinoceros* during the initial period of treatment with carbfuran. In the contrast, a significant decrease in the level of total soluble protein *Spodoptera littoralis* at all time intervals (0, 24, 48 and 96 hrs.) was recorded by El-Kordy *et al.*[37], due to the effect of Margosan-O. El-Salam[38], studied the effect of *Metarhizium anisopliae* and yeast as a biological control agent, against *Pterochloroides persicae*. The aphid individuals exhibited remarkable decrease in their total protein level with *M. anisopliae*, whereas the protein level increased with yeast. Etebari *et al.*[39], showed that in 24 h after using toxic leaves with pyriproxyfen against silkworm, the amount of total protein significantly decreased in most treatments. And after 120 h had different fluctuations and increased in some doses.

Maintenance of the balance "amino acid pool "in insects is the result of various biochemical reactions carried out by a group of enzymes called amino acid transaminases [40], such reactions are mainly responsible for the degradation and protein metabolism and the synthesis of certain specific compound. Both GPT and GOT relative activities increased after 48hrs then decreased after 72hrs post-treatment, but after 96hrs GPT increased by 108.04% while GOT decreased by 5.96% as compared to control (Fig.3). Similar results were found by El-Sheakh *et al.*[41], they found a negative relationship between insecticides and GOT where as GPT recorded a positive relationship after, insecticides application in cotton aphid *A. gossypii* (Glov.). On the contrary, Eid[42], found that different concentrations of Chloropyrifos caused increase in GOT activity, while GPT was decreased in *Spodoptera littoralis*. While, Kandil *et al.*[43], tested Dimilin and Atabron, against newly hatched larvae of the pink bollworm, *Pectinophora gossypiella* under laboratory conditions and found Larvae treated with the LC_{50} of Dimilin and Atabron increased the activities of transaminase enzymes GOT and GPT. And Mead *et al.*[44], concluded that spinosad and triflumuron either alone or as mixtures with two surfactants, Triton X-100 and Tween-20 on some biochemical response of 4^{th} instars larvae of S. *littoralis* after 24, 48 and 72 hours caused highly decreased in transaminase enzymes GOT and GPT.

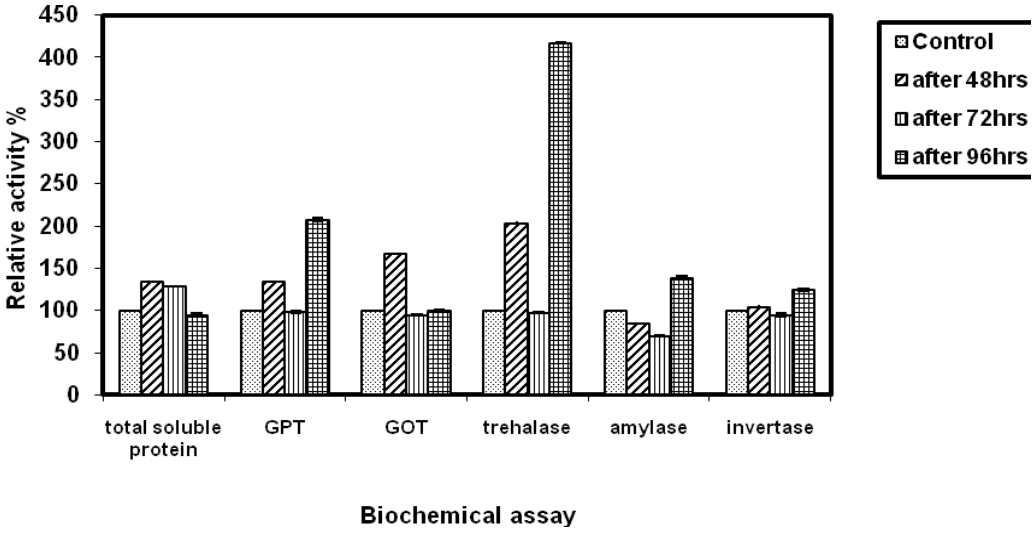


Fig (3): biochemical response of treated adult *A. gossypii* with *T. hamatum* (10^8 spores/ml) spore suspension, after 48; 72 and 96hrs post-treatment as compared to untreated (control).

Carbohydrates are contributed to the structures and function of insect tissues. Metabolism of carbohydrates controlled mainly by amylase, trehalase and invertase enzymes, which play role in the digestion and utilization of carbohydrates by insect [45,46]. Trehalase activated during molting to generate production of glucose for chitin build –up. Invertase and amylase are two important digested enzymes. In this study, it was found that, the carbohydrates hydrolyzing enzymes; amylase, trehalase and invertase of the cotton aphids were increased after 96hrs post-treatment of *T. hamatum* in 317.17, 39.24 and 24.28%, respectively as compared to control (Fig.3). Similarly, Khedr *et al.*[47], studied the biochemical effects of five insect growth regulators (IGRs) against 2^{nd} and 4^{th} instars larvae of *Spodoptera littoralis* under laboratory conditions. The tested IGRs increased

markedly the activity of trehalase, invertase and amylase in both 2nd and 4th instars after 2 and 5 days. In contrast to, Omar *et al.* [48], who evaluated the biochemical effects of Chinmix, Spintor and Biorepel compounds on larvae of pink and spiny bollworms (*P. gossypiella* and *E. insulana*). The biochemical effects of the three compounds caused decrease in the activities of invertase, trehalase and amylase.

III.3 Histological and ultrastructural studies: Many histological and ultrastructural changes in many insects due to the infection with different entomopathogenic fungi were revealed using light and electron microscopy by many investigators [49,50,51]. The untreated and fungal treated adult *A. gossypii* were histologically examined after 2 and 5 days both by light and transmission electron microscope. The microscopic examination (light and electron microscopy) of adult *A. gossypii* in this study revealed that the *T. hamatum* spore suspension brought about massive disintegration and deformation of the aphid's body and tissues. Also, it revealed the development and the colonization of the fungus inside the insect. The light microscope examination (Plate 1, A and B) of untreated and treated adult cotton aphid showing different histological changes between them. The untreated cotton aphid adult semi-thin section (Plate 1, A) showed the normal structure of the aphid's body with normal intact cuticle which clearly differentiated into epicuticle and endocuticle layers. Normal adipose tissue (fatty tissue beneath the cultice); normal gut epithelial cells surrounding lumen; normal entire salivary gland surrounded by its membrane and normal ovary tissue with embryo were also observed. The basement membrane that surrounded the all insect organs in the haemcoel of the insect also appeared normal and intact. On the other hand, the treated adult aphid semi-thin section showing many abnormalities in the insect's body as appeared in Plate (1, B). There is a disorganization of the cuticle which appeared not differentiated into its epicuticle and endocuticle layers and become black-spotted indicating direct attack of the fungus on the defense system of the insects [52,50]. In the treatment of *Spodoptera littoralis* larvae with five strains of entomopathogenic fungi, many malformations were observed in the cuticle [53]. The alterations in the host's cuticle probably through physical damages owing to the mycelium growth as well as the beginning of the sporulation process of the fungus or may be due to biochemical degradation [54,50].

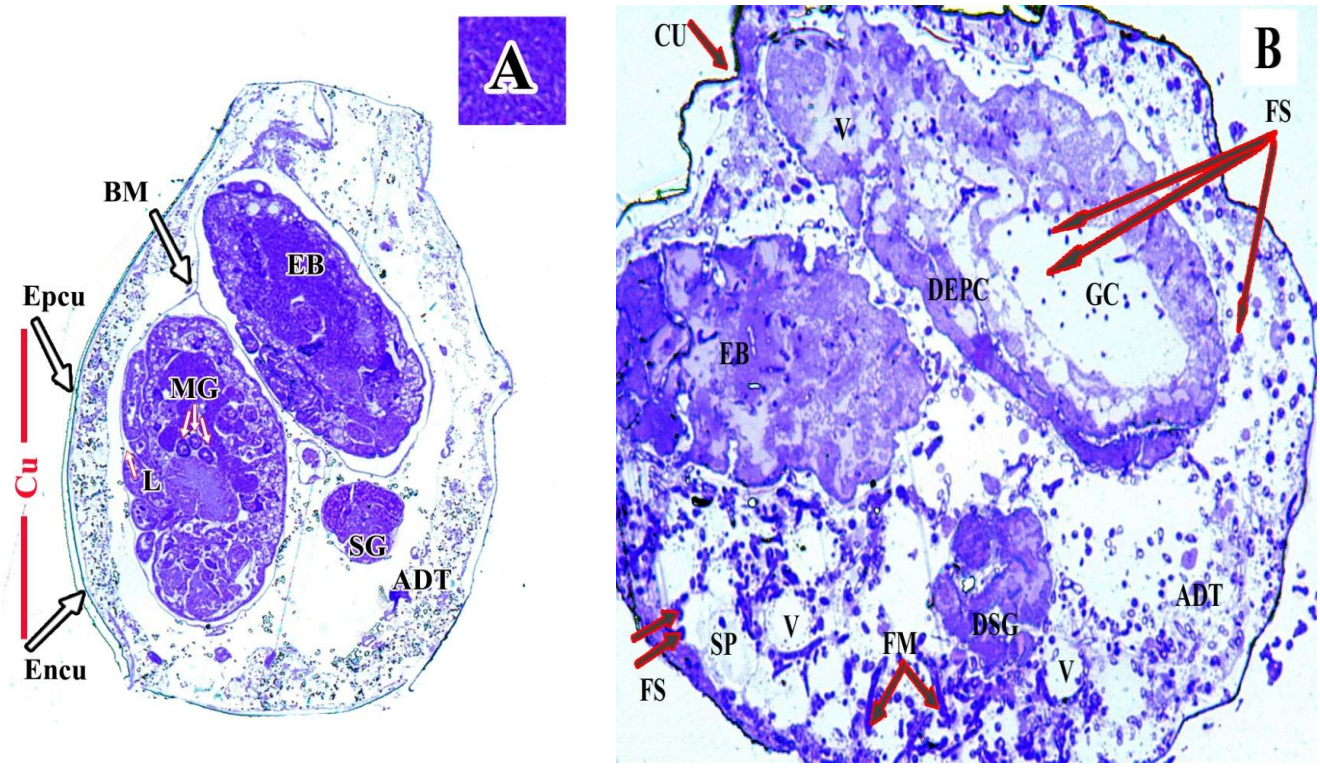


Plate (1. A and B): Light microscope micrographs of semi-thin sections of untreated adult *A. gossypii* **(A)** and treated one with *T. hamatum* (10^8 spores/ml) spore suspension **(B)**, after 5 days post-treatment. **Cu**, cuticle, **Epcu**, epicuticle, **Encu**, endocuticle, **EB**, embryo, **L**, lumen, **MG**, mid gut, **SG**, salivary gland, **ADT**, adipose tissue, **BM**, basement membrane, **DSG**, deformed salivary gland, **DEPC**, deformed epithelial cells, **GC,** gut cavity, **V**, vacuole, **FS,** fungal spores, **FM**, fungal mycelia.

The treated adult *A. gossypii* semi-thin section light microscope micrograph (Plate 1, B) showed disintegration and disappearance of the basement membrane which clearly appeared in the untreated one and surrounding the internal insect organelles. Gunnarson[55], stated that the activation of the host's innate defense

system follows upon its detection of the invading fungus via changes in the properties of the cuticle basement membrane. Also, the light microscope observations of the treated aphid showed that, there is deformation in the hind gut epithelial cells (Plate 1, B). El-Banna *et al*.[56], revealed many histological changes in the mid gut epithelial cells as a result of bacterial and viral infection of the larvae of the cotton leaf worm. The lysesof the ovarition follicles inside the ovary was also observed. The histological observations around the different ovary and embryos malformation were reported in details by many investigators [57,58,59,60]. Also, the light microscope examination of adult *A. gossypii* revealed that the salivary gland lost its surrounding membrane and appeared deformed and fragmented due to *T. hamatum* spore suspension treatment (Plate 1, B). Ultrastructural effects of crude destruxin produced by *Metarhizium* sp. on the salivary gland were reported by Sowjanya *et al*.[61], where characteristic changes including detachment of microvilli, and epithelial cell vacuolization were observed through transmission electron microscopy of *Spodoptera litura*. Also, several vacuoles were found in the body of the infected aphid and these vacuoles were more located inside the adipose tissue. The vacuoles were found in several insects parasitized with entomopathogenic fungi [62,63,64,50].

As appeared in Plate (2. C and D), the transmission electron microscope examination, revealed that, the untreated adult aphid showing its normal structure and organization as mentioned before in the light microscope examination where the cuticle appeared intact without any fungal penetration. Normal adipose tissue, mitochondria and microvilli were also, observed. Plate (3. E and F) showing treated adult aphid ultra-thin sections examined by transmission electron microscope after 2 days of treatment with *T. hamatum* spore suspension. It was observed that the fungus was able to penetrate the insect cuticle and passed through the subcutaneous muscle which appeared deformed and disintegrated. This is probably due to the fact that *T. hamatum* during its development caused separation of the muscle fibers. This observation was previously confirmed by Alves[54] and Schneider *et al*.[50]. It was also observed that the insect produced numerous secretory granules which appeared scattered inside the adipose tissue. This may be a defense mechanism of the insect against the fungus penetration. El-Banna *et al*.[56], reported that several secretory and lipid granules were observed in the internal tissues of the cotton leafworm larvae due to the bacterial infection.

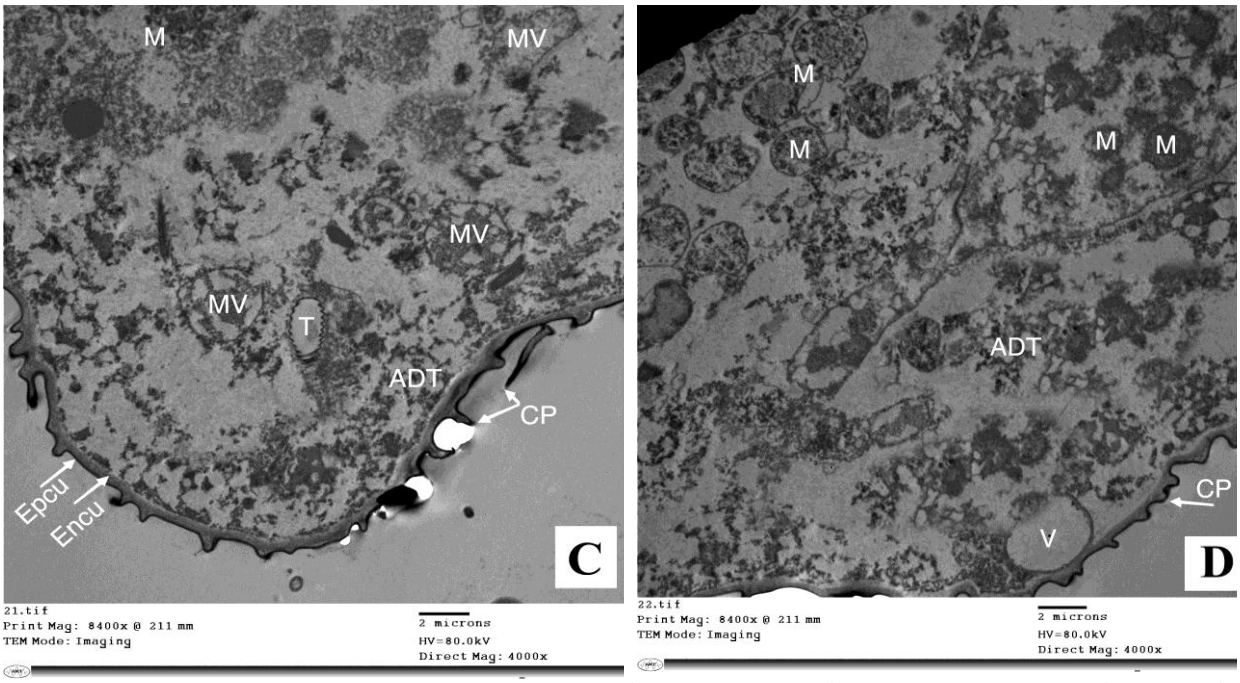


Plate (2. C and D): Transmission electron microscope micrographs of ultra-thin sections of untreated adult *A. gossypii*, after 5 days of rearing. **Epcu**, epicuticle, **Encu**, endocuticle, **ADT**, adipose tissue, **CP,** cuticle projection, **M**, mitochondria, **MV**, microvilli, **T**, trachea.

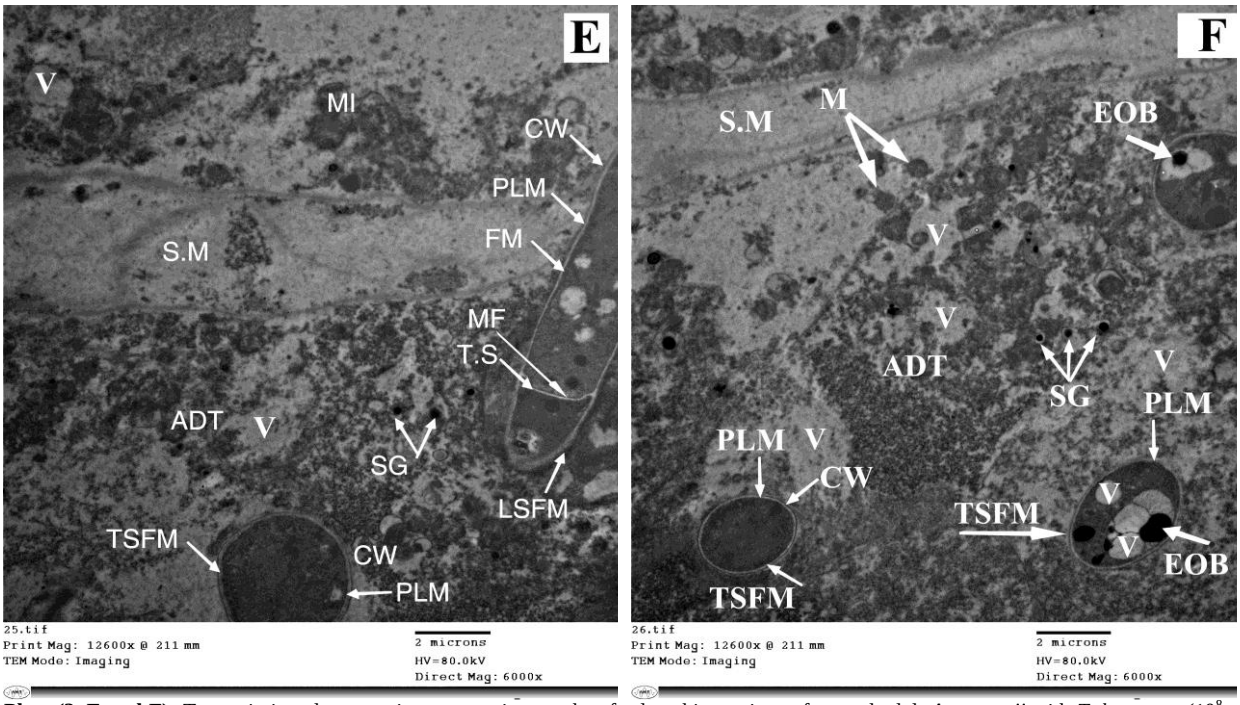
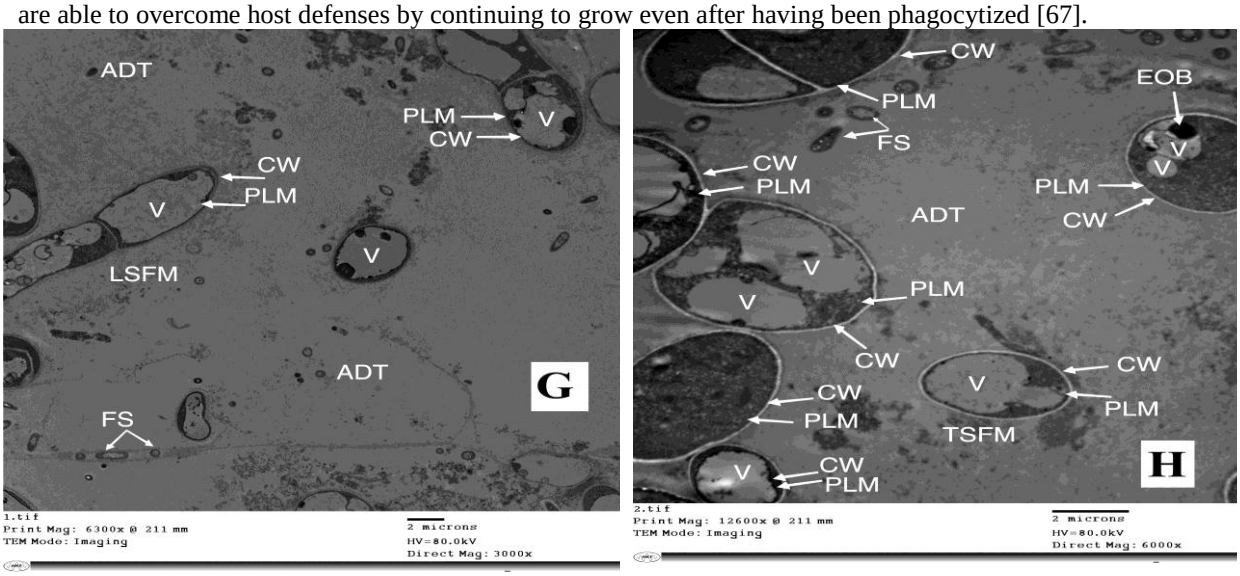


Plate (3. E and F): Transmission electron microscope micrographs of ultra-thin sections of treated adult *A. gossypii* with *T. hamatum* (10^8 spores/ml) spore suspension, after 2 days post-treatment. **ADT**, adipose tissue, **V**, vacuole, **M**, mitochondria, **TSFM**, transverse section in fungal mycelium, **LSFM**, longitudinal section in fungal mycelium, **CW**, cell wall, **PLM**, plasma membrane, **FM**, fungal mycelium, **MF**, fungal mitochondria, **MI,** insect mitochondria, **S.M**, subcutaneous muscle, **SG**, secretory granules, **EOB**, electron opaque bodies.

The *T. hamatum* colonization and development inside the aphid's body was revealed by both light (Plate 1, B) and transmission electron (Plates 3 and 4) microscope examination where, the adipose tissue which appeared disintegrated and more vacuolated, was occupied by the fungal mycelia; yeast-like hyphal bodies and spores. Death of the infected host usually occurs during the colonization of the haemocoel, where in the host suffers depletion of nutrients, or starvation, as was shown in *Culex pipiens quinquefasciatus* larvae infected with the oomycete *Lagenidium giganteum* [65]. Inside the insect haemocoel the fungus switches from filamentous hyphal growth to yeast-like hyphal bodies or protoplasts that circulate in the hemolymph and proliferate via budding [66]. The process of fungal colonization was rapid after 5 days of treatment as appeared in Plate (4. G, H, I and J). It was seen that the adipose tissue; lumen and other disintegrated insect structures and organelles were totally occupied by the fungal hyphal growth; yeast-like hyphal bodies and spores. The transmission electron microscope examination showed that the *T. hamatum* hyphae appeared as L.S. and T.S. inside the insect tissues (Plates 3 and 4). These hyphae appeared more vacuolated and crowded with several electron opaque bodies. This may be a response from the fungus against the host defense. In most cases entomopathogenic fungi are able to overcome host defenses by continuing to grow even after having been phagocytized [67].



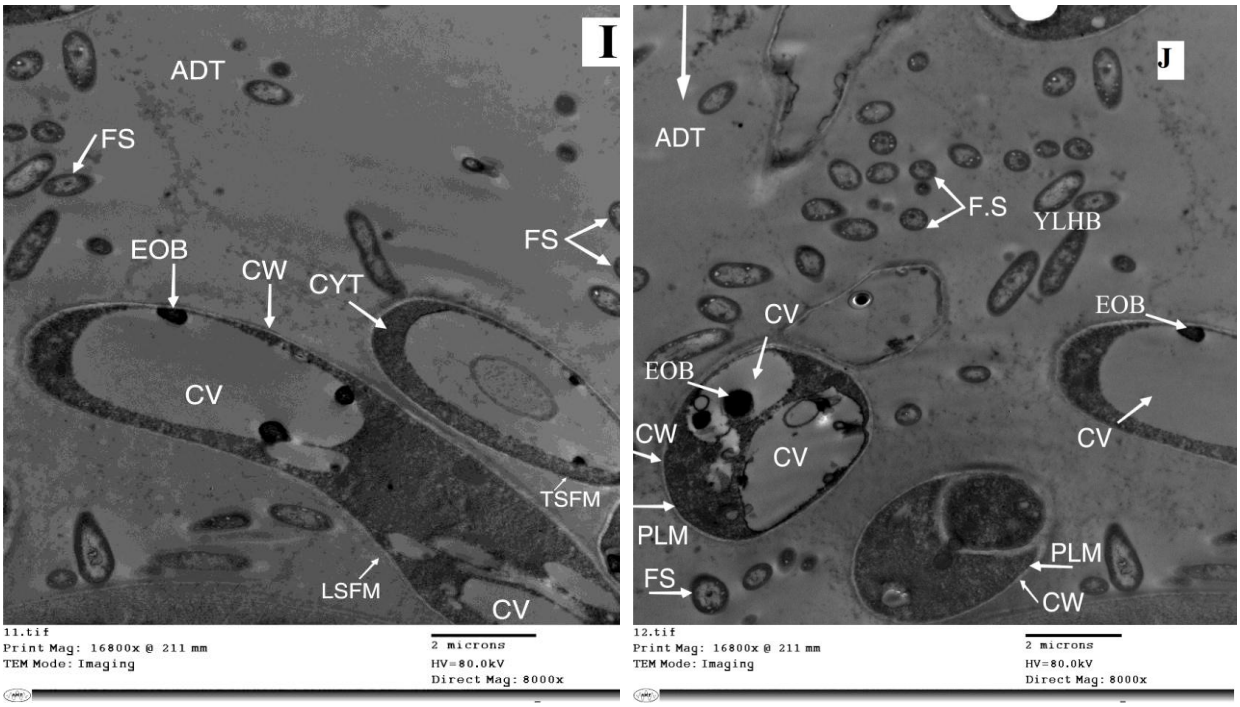


Plate (4. G, H, I and J): Transmission electron microscope micrographs of ultra-thin sections of treated adult *A. gossypii* with *T. hamatum* (10^8 spores/ml) spore suspension, after 5 days post-treatment. **ADT**, adipose tissue, **V**, vacuole, **FS,** fungal spores, **FM**, fungal mycelia, **TSFM**, transverse section in fungal mycelium, **LSFM**, longitudinal section in fungal mycelium, **CW**, cell wall, **PLM**, plasma membrane, **FM**, fungal mycelium, **EOB**, electron opaque bodies, **Cyt,** cytoplasm, **CV,** cytoplasmic vacuole, **YLHB,** yeast-like hyphal bodies.

IV. Conclusion

T. hamatum could be used as a biocontrol agent against *A. gossypii* and the morality, biochemical, histological and ultrastructural changes of *A. gossypii* as a result of *T. hamatum* infection may be caused by the physical invasion of the fungus vegetative growth and sporulation or may be also due to the enzymatic degradation or toxin production.

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