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HISTOPATHOLOGICAL AND SCANNING ELECTRON MICROSCOPE STUDIES OF ENTOMOPATHOGENIC FUNGUS (*BEAUVERIA* SPP.) ON DIFFERENT STAGES OF *MUSCA DOMESTICA* (DIPTERA: MUSCIDAE)

By
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Abstract

The entomopathogenic fungus, *Beauveria spp.* was isolated from house flies collected from different localities in various Egyptian Governorates. Flies were trapped from garbage piles, dairy and meat production processes on cattle farms and fowl farms. The study focused on *Beauveria spp*. as entomopathogens were widely used in recent decades as a biological control of the population of house flies, to avoid hazardous effects of insecticides on man animals, and natural eco-systems. Experimental laboratory trials were done using immersion of larvae on serial dilution of *Beauveria* spores reared different stages of the house flies. Infected larvae, pupae and adults were examined macroscopically for visible growth of fungi, before further studies using scanning electron microscope and histopathological methods, to investigate destructive impact of the fungus on the external and internal structures. The previous investigations revealed adhesion growth and propagation of the conidia on the cuticle of all stages, including compound eyes, setae of the legs, thorax, wings and abdomen of adults. Penetration and propagation of the conidia was evident in larval internal tissues causing lysis of fat cells, and of thoracic muscles of adults.

Keywords: Egypt, *Musca domestica*, Entomopathogens, SEM, Histopathology, Biological control.

Introduction

Since the 20th Century, parasitologists and medical entomologists have recorded the mechanical or biological transmission of different pathogens by *Musca domestica* or the house fly, Diptera: Muscidae (Farooq and Freed, 2016). House flies act as infective stages of helminthes, protozoan cysts, and bacterial agents which induce fatal diseases such as typhoid, cholera, tuberculosis, bacillary and amebic dysentery, infantile diarrhea and anthrax in man and animals (Lecuona *et al*, 2005; Förster *et al*, 2009). In recent decades, research established the serious role of *M. domestica* as transmitters of dangerous fungi types, chiefly *Aspergillus* spp. (Sales *et al*, 2002) that induce hazardous diseases such as fungal pneumonia in man and animals and nail infections in humans (Schuster *et al*, 2002; Amaike *et al*, 2011). Conventional insecticides are the primary control method of *M domestica.* However, they have created severe problems such as insecticide resistance in addition to the serious residual effects of chemicals in consumed animal carcasses. Evidence proved that houseflies were resistant to all conventional insecticide groups as organophosphates, organochlorines, carbamates and pyrethroids (Azzam and Hussein, 2002). This problem and insecticide expenses paved the way for other alternatives agents as entomopathogenic fungi as a potential biological control of insect pest line (Geden, 2012). Compared to devastating insecticide effects, entomopathogenic fungi have less hazardous impacts on global eco-systems including mammalian cycles. The presence of entomopathogenic fungi in areas and places where houseflies feed, dwell and propagate provided significant opportunities to use it to manage housefly populations (Khan*et al*, 2012)

There are many examples of successful fungus-based insect control programs by using *Beauveria bassiana* and *Metarhizium anisopliae* (Shah and Pell, 2003; Roberts and Leger, 2004). *B. bassiana* is the commonest employed fungus for house-fly control with

high mortality rates within 5-15 days.

The present study aimed to study the role of *Beauveria bassiana* as a biological control versus different developmental stages of *Musca domestica*. Stages were immersed in serial dilution of *Beauveria* spores for certain time, and reared under close observation to evaluate the fungi effect. Infected larvae, pupae and adults were examined macroscopically for fungi growth by SEM and histopathologically for exposed and non-exposed stages, to evaluate fungus destructive effect on external and internal structures in comparison with control non-exposed ones.

Material and Methods

Collection of *Musca domestica*: *M. domestica* as identified (Hafez e*t al*, 1971), were collected from garbage piles, dairy and meat processing plants and fowl farms from the Giza (Nahia and Talbia district), El-Behira, El Gharbia and Kafr El-Sheikh Governorates. About fifty house-flies were gathered from June to September 2019 at each governorate, using a sweeping net.

Rearing*:* Flies were bred under laboratory conditions of 25-28±5°C & 40-65% RH in 30×30×30cm insect-proof wooden cage. Each has three sides of narrow wire mesh and a fourth side that is vertically movable with a hole in its middle. A piece of cotton cloth was tightly fixed to the hole by adhesive glue. Cloth was closed at its terminal end by a rubber band to enable safe access to the inside of the cage. Adult flies were fed a mixture of equal parts (25gm) of powdered milk and granulated sugar, mixed with water. A piece of cotton was soaked in the mixture and put in Petri-dishes in breeding cage. Hatched larvae were transferred to 250ml. glass beakers containing a larval diet of 5 gm. wheat bran, 2gm. milk powder and 1ml. honey mixed with 10ml of water. Diet was replenished daily until the emergence of pupae. Pupae were transferred to 250ml. glass beakers with saw dust in the bottom, covered by a piece of goose, tied by an elastic band, and put in the breeding cage at 25-28±5°C & 40-65% RH.

Isolation of fungi from collected samples.

Ordinary fungal growth media: Collected flies from each locality were separated in five groups. The first group was composed of 30 flies collected from garbage piles in the Nahia District. The second group contained 25 flies from garbage piles in the Talbia district and Giza governorate. Other groups each had fifty flies from a dairy processing plant in El-Behira a fowl farm in El Gharbia and a meat processing plant in Kafr elShikh. All flies were preserved in test tubes and placed in a freezer for 5 minutes to anesthetize them. To sterilize the flies, they were washed in 1% sodium hypo-chlorite solution and rinsed twice in sterile distilled water for 3 minutes and 1 minute respectively (Seymour *et al*, 1984). The flies were transferred to a porcelain crucible for maceration, by adding a saline solution drop by drop using smooth grinding. About 0.1ml of the macerated preparation was spread on a fungal growth media containing 32.5gm. SDA (Sabouraud Dextrose Agar) in 500ml distilled water with 0.025gm.

Chloramphenicol was used as antibiotic to inhibit bacterial growth and 0.125gm cycloheximide as saprophytic fungicide. Five plates of the prepared fungal growth media were used for each flies, group. Plates were stored at 28°C±1 & RH of ≥80%. Daily observations for 15 days to observe fungal colony growth. Isolated colony was macroscopically identified by specific color and shape and examined microscopically (Sales *et al,* 2002).

Growth media for entomopathogenic fungi: To isolate lethal from non-lethal fungi usually present in adult fly bodies, 0.1ml of the macerated preparation was cultivated into the selective media specific for entomopathogenic fungi, *Beauveria* and *Metarhizium* species. Media consisted of 32.5gm SDA suspension (Sabouraud Dextrose Agar) in 500ml distilled water with 0.025gm chloramphenicol, added to 0.1gm of selective substance, dodine (Zarrin *et al*, 2007), as an inhibitor to the other saprophytic fungi.

Infection of *M. domestica* larvae: To infect larvae with the isolated entomopathogenic fungus, spore suspensions were prepared by adding 10ml of distilled water and 0.05ml of 0.5% tween 20 to the fungal colony plates. Fungal spores were counted by a hemocytometer using 10µm of spore suspension (Farooq and Freed, 2016). Serial concentrations of 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} spores/ml were prepared.

To study cumulative lethal effects of the isolate on different stages of the flies, forty larvae of 2nd stage were infected by dipping them for 10 seconds in each concentration. Three larvae replicas infected by each spore concentration were used. Control groups of larvae were prepared by dipping them in a solution of a 0.05% Tween 80 only. Larvae were transferred to small plates containing larval diet, and incubated at 25±5°C & 65% RH, for 4 daily observations, to count them. From 5th day onward, larvae were checked daily for pupal emergence, which relocated to a glass beaker containing a layer of sawdust. The emerged adult flies were checked daily for 14 days to count the mortality percentages resulting from the fungi.

Infection of adult M. *domestica*: Forty flies from fungal spore concentrations of 10^5, 10^8& 10^{10} were used for each infection trial. A patch of same flies number was used as a control by immersing them into a solution of 0.05% Tween80 only. All infected and control groups were stored and fed as previously described and were observed daily for 14 days for mortality recorded.

The cadaver was daily collected and put on moist filter papers in Petri-dishes to facilitate fungal sporulation (Farooq and Freed, 2016). Mortality resulting from fungal infection was identified by examining the fungal growth color and shape, indicative for each specific type of entomopathogenic fungi (Steinkraus *et al*, 1990). For more confirmation, the cadaver were washed in 10ml sterile distilled water, stirred for 30 seconds to extract spores, and examined under a light microscope (Mwamburi *et al*, 2010).

A histopathological study investigated the entomopathogenic fungus effect on internal and external tissues of the different stages. Five samples from each group of infected larvae and adults with fungal concentration of 10^{10} were fixed in 10% formalin. Then, they were washed in water, dehydrated in ethanol, cleared in xylene and embedded into paraffin wax (Bancroft *et al*, 1996). By standard histological protocols, sectioning (5-10 µm) were put on slides and stained with hematoxylin and eosin (H&E). Masson's trichrome (MT) and periodic acid Schiff (PAS) were also used (Toledo *et al,* 2010)

SEM: Samples from different stages of infected and control larvae, pupa and adult with fungal concentration of 10^{10} only were fixed in 2.5% glutaraldehyde of PH 7.2 buffer for 6hrs, and then fixed in 1% Osmium tetroxide (OsO_4) for 1hr. Larvae were dehydrated in ascending ethanol series, dried in CO_2 critical drier (Autosamdri-815, Germany) and glued over stubs, coated with gold, examined and photographed using SEM (JSM 5200, Electron Microanalyzer, Jeol, Japan) in a SEM Center, Faculty of Agriculture, Cairo University.

Results

Thorough histopathological investigations were implemented to investigate destructive effects of naturally isolated entomopathogenic fungus, *Beauveriaspp* on the different stages of *M. domestica.* Two special stains, Masson trichrome (MS) and Periodic Acid Schiff (PAS) stains were used in addition to the H&E stain. The MS stain functions as an identifier of the internal tissues of dipterous flies. The PAS stain effectively in defines the hyphae, and spores of the fungus, which stained in purple–red, counter stain of the flies' tissues that colored dark blue.

Histopathology of infected larvae: Control larvae stained by the PAS stain showed normal outer structures such as, cuticular integument, muscular system and cephalopharyngeal region without any signs of fungal spores and/or hyphae presence. In contrast to the controlled ones, infected larvae stained

by PAS showed degenerative lesions including lysis of adipose tissue in tbody cavity. Also, stained larvae by PAS and MT showed fungal spores in cuticular integument, beside its penetration of the body cavity.

Histopathology of infected adults: Adults of non-infected stained by PAS showed normal external structures including head, thorax, abdomen and compound eyes. Internal tissues like testes and Malpighian tubules were also intact. But, 24hrs postmortem females by 10^{10} concentrated fungal spores stained by MT exhibited aggregations of fungal spores in abdominal region near hindgut and vagina.

The present study showed that over time there was a severe destructive impact of the fungus on the outer and inner structures of adult flies. In 72hrs postmortem adults stained by MT stain, exhibited muscular lysis in the thoracic region. By using MT stain, fungal were clearly aggregated in the compound eyes. The previous facts worsened in case of 10 days cadavers, where all parts of the outer cuticle include compound eyes, thorax, legs and abdomen were mushroomed by hyphae and fungal spores. In sections those stained by PAS & MT, the proboscis was attacked by fungal spores, while control cadaver stained by H&E and MT showed normal legs, muscle and head.

SEM of infected larvae, pupae and adults of *M. domestica* by 10^{10} spore concentration: SEM images of *M. domestica* control larvae showed normal mouth parts with intact oral hooks. In contrast, infected two-day larvae with *Beauveria* spp. by 10^{10} spore concentration, showed severe destruction of oral hooks, and cuticular papillae surrounding mouth parts. But, control larvae showed normal and intact cuticles, smooth and well-formed body parts, fungal spores on the larval cuticle exhibited severe dehydration, accompanied by cuticular destruction.

In the 14- day post infection pupae, varied mycelia growth stages and numerous fungal spores were observed on magnified surface of infected pupa. No abnormal signs on the pupae longitudinal view were observed. By scanning examination, two-day infected adult flies showed severe com-pound eye destruction with fungal spore aggregation in different parts of compound eye, maxillary bulb, labella and antenna. Spores mushroomed were on adults compound eye 6hrs post emergence. Germ tubes and fungal spores adhering to ommatidia were clearly seen in 48hrs post emergence adults. Germ tubes were detected on thorax dorsal surface and legs including tarsus region. Dorsal surface of abdominal region showed a heavy mycelium network with massive aggregations of conidia. In contrast, control flies appeared devoid of any signs of infection like spores or mycelium growth either in head, thorax or abdomen. The detailed were given in figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 & 12)

Discussion

Entomopathogenic fungi invaded the host cuticle shortly after its germination, or after limited hyphal growth (Leger *et al,* 1991). Fungal infection begins when conidia attach to the insect cuticle; the spores germinate, penetrate the insect cuticle and go through the host. Once the fungus penetrated host, it produces toxins and secondary metabolites that overcome the insect immune system to grow rapidly (Roberts, 1981). In the present study, this fact explained the destruction of all stages of *M. domestica* infected by high spore *Beauveria* spp. concentrations. The sections showed lethal effectiveness on internal and external tissues of the flies. Formation and multiplication of hyphal bodies by *Bea-0uveria* spp. inside and outside the host body was marked. In case of 24hrs postmortem of adults, hyphal bodies and spores were seen in abdominal region near hindgut and vagina. The 72hrs cadavers showed that the entire body was invaded by fungal spores, whereas the thoracic muscles showed a clear lysis, and the fungal spores obviously attacked the flies' ommatidia. This finding agreed with Toledo *et al*. (2010) who found that highest concentration of spores and hyphal bodies were detected in compound eyes

and the abdomen terminal region with lysis of the body fat cells and muscular tissue.

The present SEM showed that conidia of *Beauveria* spp. attached itself to all body regions of infected flies, particularly areas of dense hair cuticle. This agreed with Boucias *et al*. (1988), and Boucias *et al*. (1991). In this case, *Beauveria bassiana* conidia were abundantly in the hair of infected 4th-instar larvae of *Velvetbean caterpillar* (*Anticarsia gemmatalis*). *Beauveria* spp conidia were between ommatidia of compound eye and at articulating membrane of adults' legs. This agreed with Hasaballah, *et al.* (2017) who recorded *B. bassiana* and *Metarhizium anisopliae* conidia on compound eye, thorax, legs and abdomen of *M. domestica*. Toledo *et al.* (2010) found that conidia of *B. bassiana* isolate biologically controlled *Peregrinus maidi* and attacked its compound eyes and legs.

Conclusion

The present study succeeded in isolating of the entomopathogenic fungus, *Beauveria spp.* from *Musca domestica*. SEM and histopathology studies proved that the fungus has destructive effects on external and internal structures of the larvae, pupa and adults of house flies. Thus, fungal spore is recommended for biological control of house flies.

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Explanation of figures

Fig. 1: Histopathology of control and infected larvae. (A): Control larva stained by PAS showed normal structures, cuticular integ ument (ci), muscular system (ms) and cephalophryngeal region (cp) spores. (B): Infected larva stained by PAS showed degenerated tissue (dt) lysis of adipose tissue (at). (C): Infected larva stained by PAS showed fungal spores in outer surface and body cavity (fs). (D): Infected larva stained by MT showed fungal spores in outer surface of larva (fs).

Fig. 2: Histopathology of control and 24hrs postmortem adults. (A): Control female stained by PAS showed normal structures, head (h), thorax (th) and abdomen (ab). (B): Control adult male stained by H&E showed normal testes (t) and Malpighian tubules (mt). (C): Infected female stained by MT showed aggregation of fungal spores (fs) in abdominal region of vii-ix abdominal segments; illustrate hindgut (hg) and vagina (v). (D): Higher magnification.

Fig. 3: Histopathology of 72hrs postmortem after death adults. (A): Female stained by MT showed muscular lysis (ly) in thoracic region and fungal spores (fs) in the compound eyes. (B): Female stained by MT showed fungal aggregation (fs) in the compound eyes. (C): Higher magnification of infected adult male stained by MT showing lysis (ly). (D): Higher magnification of female stained by MT showed clear lysis area in muscle of thorax (ly) .

Fig. 4: Histopathology of control and cadaver of 10 days post-infection. (A): Control dead adult of 10 days stained by H&E showed normal leg (l) and muscle (m). (B): Control dead adult of 10days stained by MT showed normal leg (l) and head region (h). (C): Infected cadaver of 10days stained by MT showed rounded fungal spores (fs) in autolysis tissue (D): Infected cadaver of 10 days stained by PAS showed rounded fungal spores (fs) in proboscis (pr).

Fig. 5: Ten-day cadaver showed fungal spreading in all insect parts.

Fig. 6: Two-days control larva. (A): Mouth part showed normal structure with intact oral hooks (oh) and papillae (p) (X100). (B): Two -days infected larva with *Beauveria spp*. showed severe destruction (ds) of mouth parts , oral hooks (oh) and papillae (p) (X75).

Fig. 7: Two-day control larva. (A): Inter-segmental region (It) of larvae showed normal and smooth cuticle, and well-formed body parts (X35). (B): Two-day infected larva showed shrunken intersegmental cuticle (It), extreme dehydration and severe destruction (ds) of cuticle with fungal spores (fs) on body surface (X35).

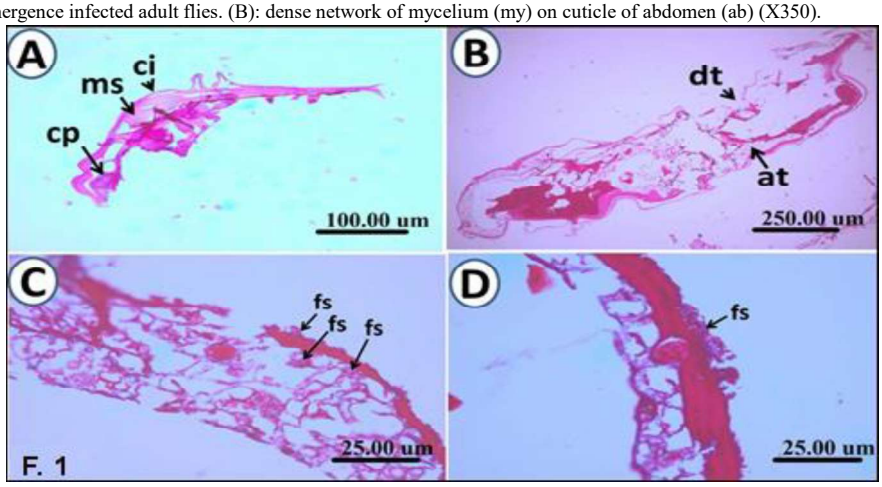
Fig. 8: One-day control *M. domestica*. (A): whole view of pupa showed normal intersegmental spines (X35).(B): Infected 14-days-pupa with *Beauveria spp*. showed varied stage of mycelial growth (my) and fungal spores(fs) (X350).

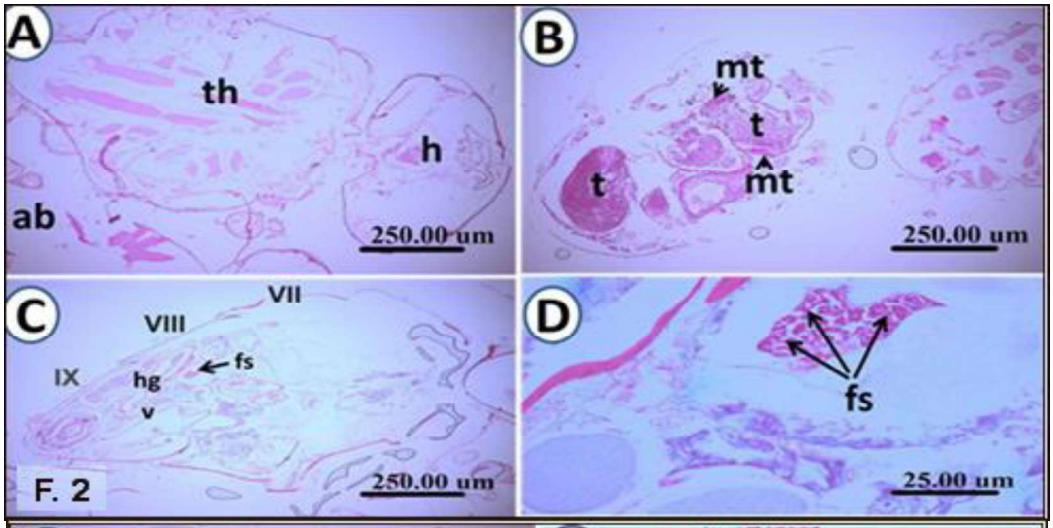
Fig. 9: Control adults of 48hrs post emergence. (A): head showed normal and intact compound eye (E), maxillary bulb (mb), labella (la) & antenna (a) (X35). (B): infected adult of 48hrs post emergence, showed severe destructions(ds) of compound eye(E) & mouth parts with aggregations of fungal spores(fs) in head different parts (compound eye(E), antenna(a) and labella) (X35).

Fig. 10: Infected adults with *Beauveria spp. (*A): 6hrs post emergence showed fungal spores (fs) adhering to ommatidia (X750). (B): 48hrs post emergence showed germ tubes (gt) and fungal spores (fs) adhering to ommatidia (X1000).

Fig. 11: Control of 48hrs post emergence adults. (A): legs (l) no destruction (ds) or mycelium growth (my) (X35). (B): 48hrs post emergence infected adults; showed a network of mycelium (my) and fungal spores (fs) on leg (l) (X150).(C): Germination and penetration of germ tube(gt) on thorax(th) (X1000).

Fig. 12: SEM of control of 48hrs post emergence adults. (A): abdomen (ab) showing normal cuticle without fungal spores (fs) (X35). 48 hours post emergence infected adult flies. (B): dense network of mycelium (my) on cuticle of abdomen (ab) (X350).

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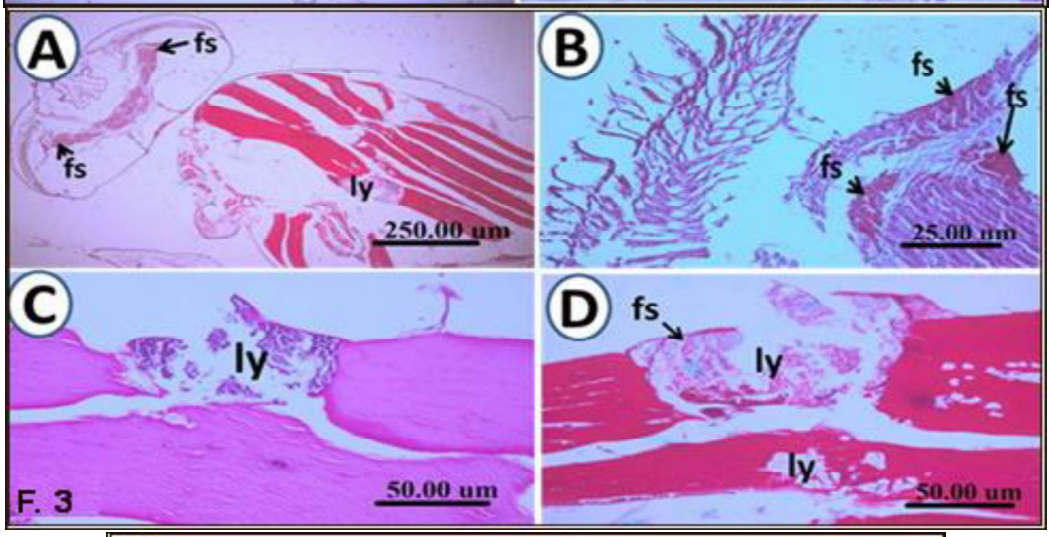
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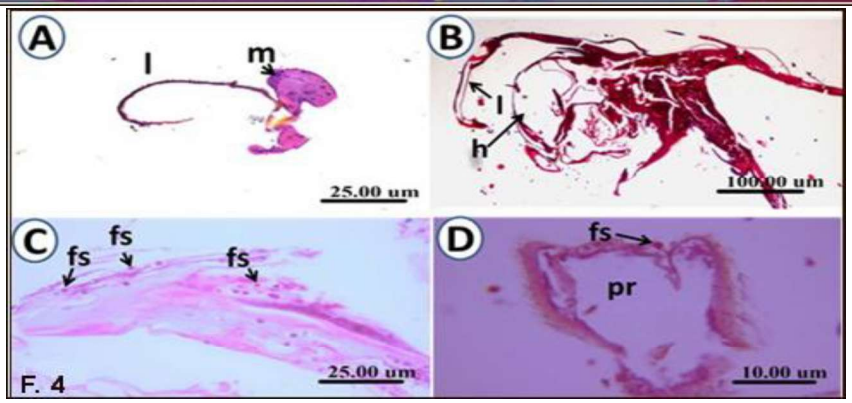
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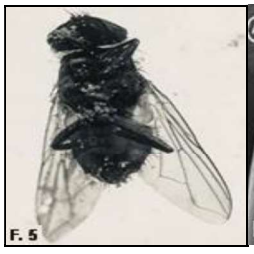
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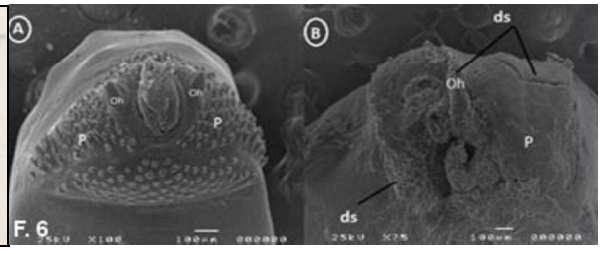
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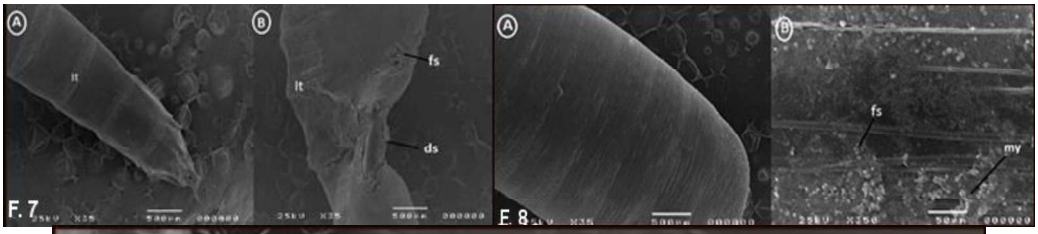

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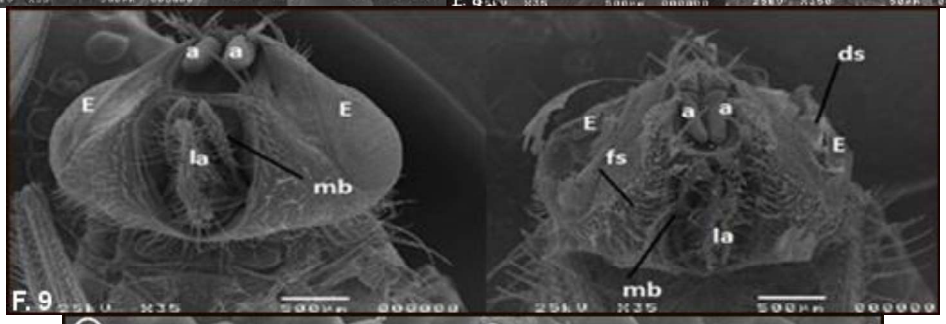

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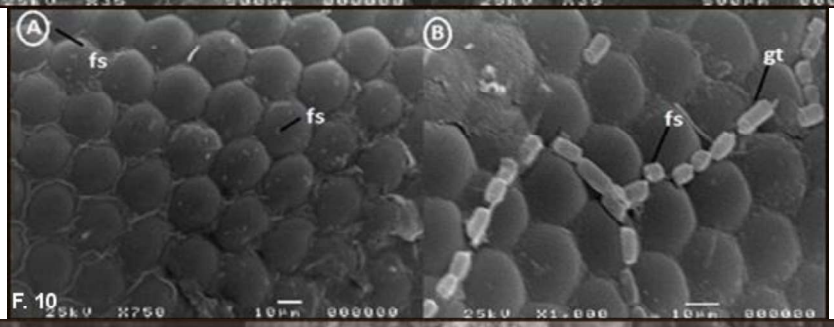
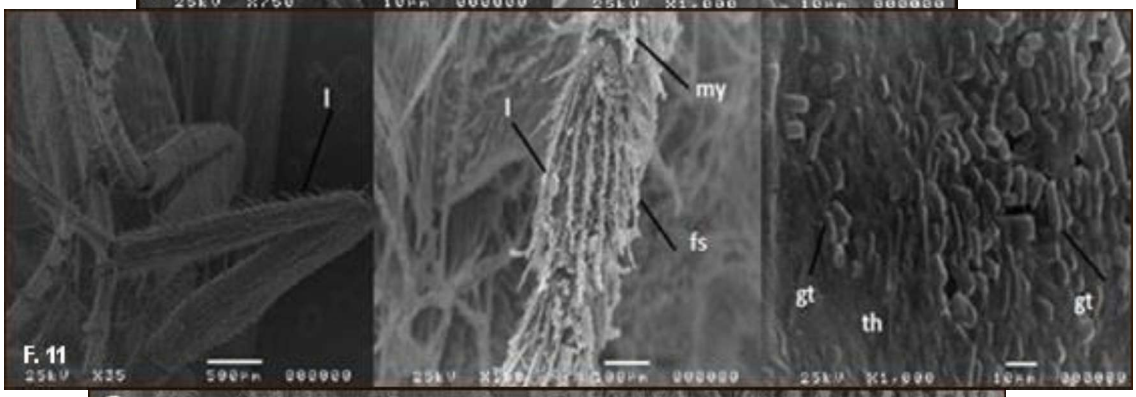
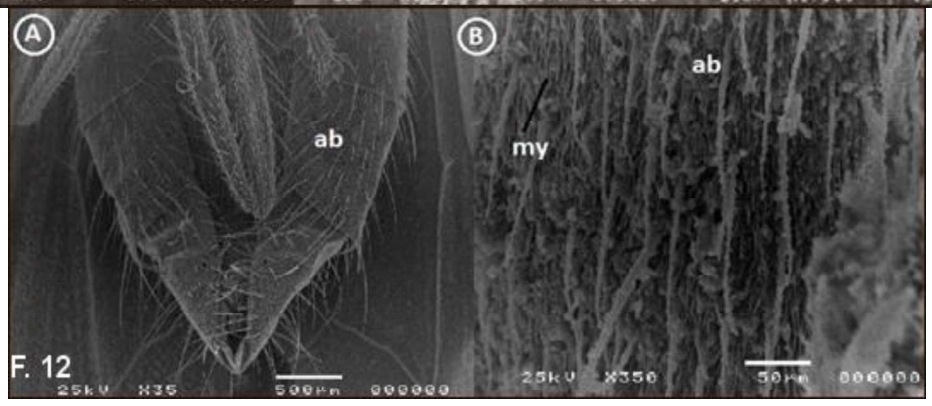


F. 10



F. 11



F. 12