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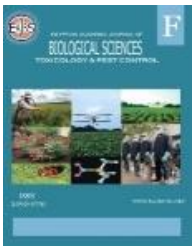

Toxicological Studies and Histopathological Changes on Black Bean Aphid, *Aphis craccivora* Induced by Entomopathogenic Fungi, *Metarhizium anisopliae* and *Purpureocillium lilacinum*

Shimaa M. Desoky[1], Yasmein A. EL Sayed[2] and Suzan A. Ibrahim[2]
1-Botany and Microbiology Department, Faculty of Science, Suez University, Egypt.
2- Plant Protection Research Institute Agriculture Research Center Dokki Giza Egypt*
Email: shemoo_1000@yahoo.com

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ABSTRACT

Toxicological, histopathological and ultrastructure studies of *Metarhizium anisopliae* and *Purpureocillium lilacinum* infected *Aphis craccivora* nymph were investigated in this study. comparison between fungal spore suspensions and fungal culture filterates virulence effects of the two tested entomopathogenic fungi against *A. craccivora* nymph were done under laboratory condition and the toxicological bioassay results revealed that *P.lilacinum* has a high toxic effect than *M. anisopliae* in which the LC_{50} of fungal spore suspensions and culture filterates after 7 days post-treatment was (7×10^2, 1×10^5 spore/ml and 3.2, 4.7 %) for *P. lilacinum* and *M.anisopliae,* respectively. Treated nymph with spore suspensions was examined using both light and transmission electron microscope to study histopathological and ultrastructure changes compared with the control one. This comparison suggested that there is a deformation of the nymph body, complete destruction of all internal organs with an extensive invasion of the fungal spores throughout all internal body regions.

INTRODUCTION

The black bean aphid, *Aphis craccivora* (Koch), spreads around the worldwide and feeds on ~ 200 host plants in diverse families (Muller, 1982). This pest is one of the most harmful of broad bean, Vicia faba, in numerous areas. *A. craccivora* can acquire very high populations, especially when the invasion of the crop happens before blossom. Thus, some plants can be destroyed and heavy yield loss may happen by direct feeding (Birch, 1985). Aphids directly damage the plant by removing the plant sap, which includes necessary nutrients that stimulate plant growth and aphid. Because bark extract is wealthy in sugars than amino acids that need for aphids prosperity. A lot of the sap is secreted as honeydew. The honeydew, sugar-rich, could screen the leaf surface by composing the suitable surface for the growth of mould fungi when aphid populations are very huge, this layer affects the impact of the pods generated. This mould fungi lessen the efficiency of respiratory, plant photosynthesis, and thus the ultimate yield. In addition, the activity of direct feed, black bean aphid can transmit more than 42 persistent and non-persistent plant viruses (McKinlay, 1992).

Microbial control refers to the biological repression of insect pests with insecticides such as viruses, fungi, bacteria, protozoa, and nematodes. Microbial biocontrol agents (MBCAs) can overcome the pest population without any dangerous influence on human health and the environment. This serves as an attractive surrogate technique to chemical pesticides (Khan *et al* 2012). The currently available MBCAs are handled worldwide with great benefits and success. The fungal biocontrol agents are the most significant among all the recognized MBCA's owing to their accessible delivery, developing formulation, an enormous number of pathogenic strains recognized, simple engineering methods and over-expression of endogenous proteins or exo-genous toxins (St. Leger *et al* 1996a, Butt *et al* 2001 and St. Leger and Wang 2009).

These mycopesticides fundamentally depend on propagules such as conidia, hyphae or blastospores. These propagules have good features of the direct homicide of the objective pest as well as secondary infection using horizontal transmission of spores from fungal carcasses (Shan and Feng 2010). Also, can produce secondary metabolic compositions that may be toxic to insects (Vey *et al* 2001). Consequently, these mycopesticides have different insecticidal activity.

The present investigation aimed to clarify the ability of *P. lilacinum and M. anisopliae* to control one of the most serious pests of agriculture crop and plant viral vector, the black bean aphid *A. craccivora* in its nymph stage and investigate the histopathological effects using light and TEM .

MATERIALS AND METHODS

The Tested Insect:

The black bean aphids (*A. craccivora*), were collected from Faba bean greenhouse in Qaha, Plant Protection Research Institute, Agriculture research center, transferred to the laboratory and reared on Faba bean leaves at 25ºC±2 and 70%±10 relative humidity.

The Microorganisms:

M. anisopliae and *P. lilacinum* were isolated from red palm weevil, *Rhynchophorus ferrugineus*, and soil sample respectively. The weevil insects cadavers were dipped in 70% ethanol, then put individually on the petri dishes contain Dox media to allow the growth of their fungal content, these fungi purified by contentious inoculation on Dox media, then examined by light microscope, while the soil sample was dissolved in distilled sterilized water, then injected on the prepared Dox medium plates and incubated at 27ºC for 72 hours. after that, the growth of fungi appeared on the media plates**,** and then purification steps were repeated till the appearance of clear and visible growth of fungi. *M. anisopliae* and *P. lilacinum* isolates were molecular identified and registered in the gene bank with the code number MT 102079 and MT 102250, respectively.

Bioassay Test:

Fungal spore suspensions prepared by the cultivation of the two tested entomopathogenic fungi on Dox media plates at 27ºC. The spores were harvested from 10 days cultures by adding 20 ml sterile distilled water containing 0.02% Tween 80 to agar plates and scraping the surface of the culture with a sterile loop. The resulted suspensions were shaken well and filtered through cheesecloth, the number of spores in the suspensions were estimated by Neubauer haemocytometer (Alves and Moraes,1998). Five concentrations (10^9, 10^8, 10^7, 10^6, 10^5 spore/ml) were prepared from the mother suspension by serial dilution to be used in the bioassay experiment, while fungal culture filtrates were prepared by inoculation the two tested fungi on Dox agar media for 10 days at 27ºC, about 6 cm diameter of grown mycelium was removed from the culture and

transferred into 500 ml flask containing 200 ml of Dox broth media, then incubated at 27^{o}C for 15 days. The filtrates were obtained by passing the liquid media through cheesecloth, and five concentrations (100, 75, 50, 25, 10%) were prepared to be used.
Laboratory bioassay was performed by leaf dipping technique (Bacci *et al*., 2009). Leaves of faba bean were dipped in the previous spore suspensions and culture filtrates of the two tested isolates for 10 seconds then left to dry at room temperature, the treated leaves were put separately in plastic boxes (10 nymphs per box). Then the leaves coated using moistened filter paper. Each concentration was replicated three times. Faba bean leaves were soaked in distilled water containing 0.02% Tween 80 as control.
Inspections were conducted 2, 5, 7 days post-treatment with recording the sum of living and dead individually. The percentage of mortality was estimated according to Abbott's equation (Abbott, 1925). LC_{50} was calculated according to (Finney, 1971) using Ldp line software (Bakr, 2000)

Observation of External Symptoms:

The external symptoms of the untreated and treated nymph by fungal spores and fungal culture filtrates were examined by binocular microscope and the photographs were taken by using a digital camera.

Histological and Ultrastructure Studies:

Black bean aphids were infected with LC_{50} of the two tested isolates to be prepared to study their histological structure and compared them with healthy ones using light and transmission electron microscope. Healthy and infected aphids were fixed in 5% glutaraldehyde and 1% osmium tetroxide as secondary fixation, then dehydrated through different concentrations of ethanol, then immersed in an epoxy resin. Semithin sections were formed with a thickness around 1 µm thickness, stained with a toluidine blue stain, and investigated using a light microscope. Ultra-thin sections were prepared with thickness around 50-80 nm, stained with uranyl acetate followed by lead citrate, then investigated using Transmission Electron Microscope, a JEOL-JEM 1010, with 80 KV at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University (John and Lonnie ,1999).

RESULTS AND DISCUSSION

External Symptoms of Treated Nymph with Fungal Isolates:

Microscopic examination of the aphid nymph treated with the two tested fungal spore suspensions showed the beginning appearance of fungal hyphae on the nymph body surface after 5 days post-treatment, these hyphae grew rapidly and covered the nymph body completely after 7 days post-treatment. Also, there were external symptoms observed on the aphid nymph body treated with two tested fungal culture filtrates summarized in shriveled, burned, shrinking, and complete distortion of the nymph body (Fig. 1).

Bioassay Test:

Many entomopathogenic fungi have been found effective versus a wide range of insect especially sucking insects pest species (Pedrini, *et al*., 2007, Van *et al*., 2007, Majeed *et al* 2017, Mustu *et al*., 2015, Amnuay *et al.,* 2013 and Yun *et al*., 2017). Our laboratory bioassay results revealed that fungal spore suspensions and culture filtrates of *P. lilacinum* have a higher toxic effect against aphid's nymph than spore suspensions and culture filtrates of *M. anisopliae*. The five treated spore suspensions and culture filtrates concentrations of *P. lilacinum* caused mortality percentage ranged from 73.3 to 96.6% after 7 days post-treatment, while the five *M. anisopliae* spore suspensions and culture filtrates caused mortality percentage ranged from 50 to 96.6% and from 66.6 to 96.6%

respectively after the same days (Tables 1&2). A linear relationship between tested spore suspensions and culture filtrates with mortality percentage was made by LDP line program to calculate LC_{50} after 2, 5, and 7 days post-treatment (Figs. 2, 3, 4 and 5). Lower LC_{50} (7×10^2 and 1×10^5 spore /ml) was obtained after 7 days post-treatment for *P. lilacinum* and *M. anisopliae* spore suspensions respectively and 3.2 and 4.7% for the two fungal isolates culture filtrates respectively after the same days mentioned above.

These results proved that our two isolates (spore suspensions and culture filtrates) induced highly mortality percentage of black bean aphid, and these are in agreement with (Van *et al*., 2007), who tested the efficacy of twelve strains of entomopathogenic fungi in aphid control and found that *M.anisopliae* have the highest virulent pathogenicity for both *Myzus persicae* and *A. gossypii*. Also (Khaleil *et al.*, 2016) tested four concentrations of *Trichoderma hamatum* against *aphid gossypii*, noticed the efficacy of this fungal isolate, and concluded the ability of this isolate to be used as a biocontrol agent against *A. gossypii*.

Our results also are in harmony with (Nazir *et al*., 2019) who tested the pathogenicity of two *Beauvaria bassiana* strains and one strain of *Lecanicillium lecanii* against green peach aphid *Myzus. persicae* and proved the effectiveness of these strains in which the two *B. bassiana* strains exhibited high mortality percentage reached 95% and 87% mortality percentage induced by *L. lecanii* strain.

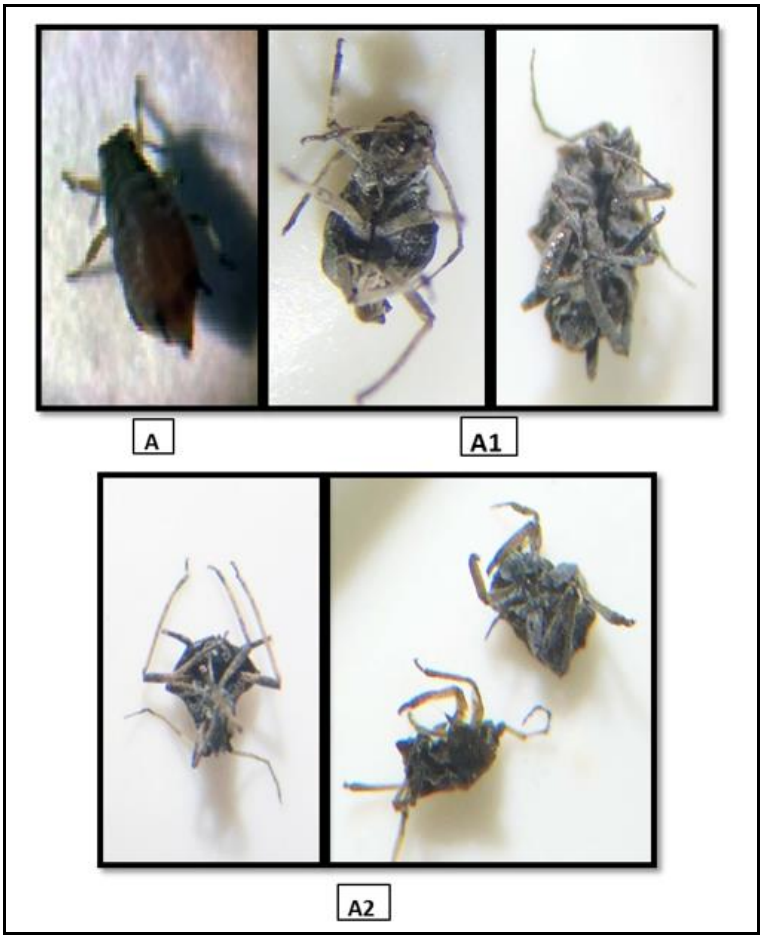

Fig. 1: A: Untreated black aphid bean showed normal body structure. **A1**: the hyphae grew around the body marginal regions with completely covering the whole nymph body surface. **A2:** shriveled, shrinking, and a complete distortion of the whole nymph body surface.

Table 1: Mortality percentage and LC_{50} value of five concentrations *M. anisopliae* and *P. lilacinum* spore suspensions against aphids nymph after 2, 5, and 7 days.

concentration. (Spore/ ml)	Mortality percentage of *M.anisopliae* spore suspensions			Mortality percentage of *P. lilacinum spore* suspensions		
	2 days	5 days	7 days	2 days	5 days	7 days
10^9	66.667	86.667	96.667	70	96.66	96.66
10^8	60.000	66.667	90	60	80	96.66
10^7	56.667	60.000	70	40	70	90
10^6	36.667	43.333	60	20	60	83.33
10^5	10	30	50	20	40	73.33
LC_{50}	2×10^7	2×10^6	1×10^5	3×10^7	4×10^5	7×10^2
Upper limit of LC_{50}	1×10^8	1×10^7	7×10^5	2×10^8	1×10^6	2×10^4
Lower limit of LC_{50}	5×10^6	4×10^5	1×10^4	1×10^7	4×10^4	9×10^{-4}

Table 2: Mortality percentage and LC_{50} value of five concentrations ***M. anisopliae*** and *P. lilacinum* culture filtrates against aphids nymph after 2, 5, and 7 days

Concentration (%)	Mortality percentage of *M.anisopliae* culture filtrates			Mortality percentage of *P. lilacinum* culture filtrates		
	2 days	5 days	7 days	2 days	5 days	7 days
100	36.667	80	96.667	90	96.66	96.66
75	26.667	60	90	80	93.33	96.66
50	16.667	40	83.333	80	93.33	93.33
25	10	30	76.667	60	80	86.66
10	3.333	30	66.667	50	66.66	73.33
LC_{50}	438.4	43.8	4.789	11.594	4.919	3.236
Upper limit of LC_{50}	-	70.18	10.8111	19.445	10.17	7.99
Lower limit of LC_{50}	-	26.4693	0.2592	3.2193	0.634	0.1106

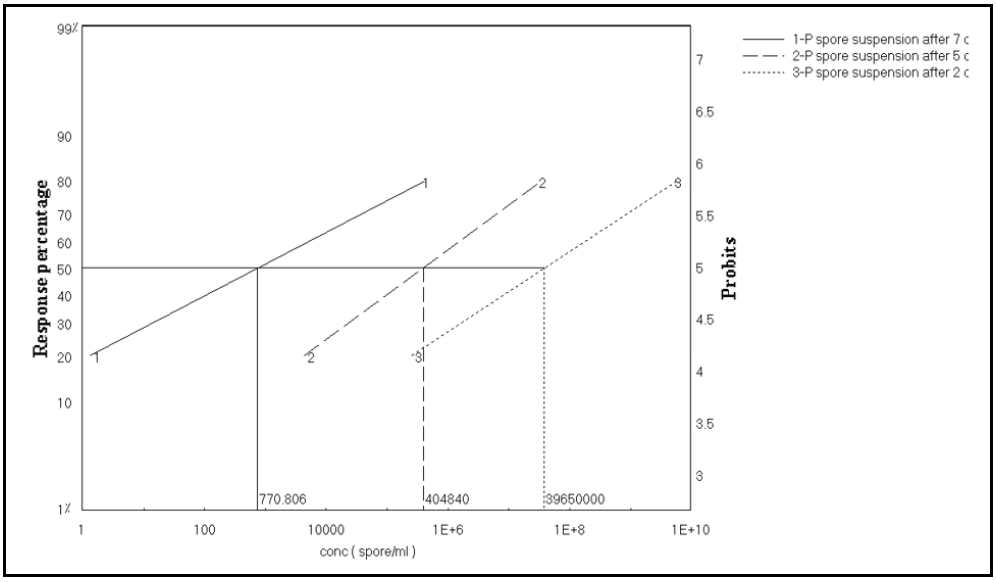

Fig. 2: The mortality percentage and five concentrations of *P. lilacinum* spore suspensions linear relationship after 2, 5 and 7 days.

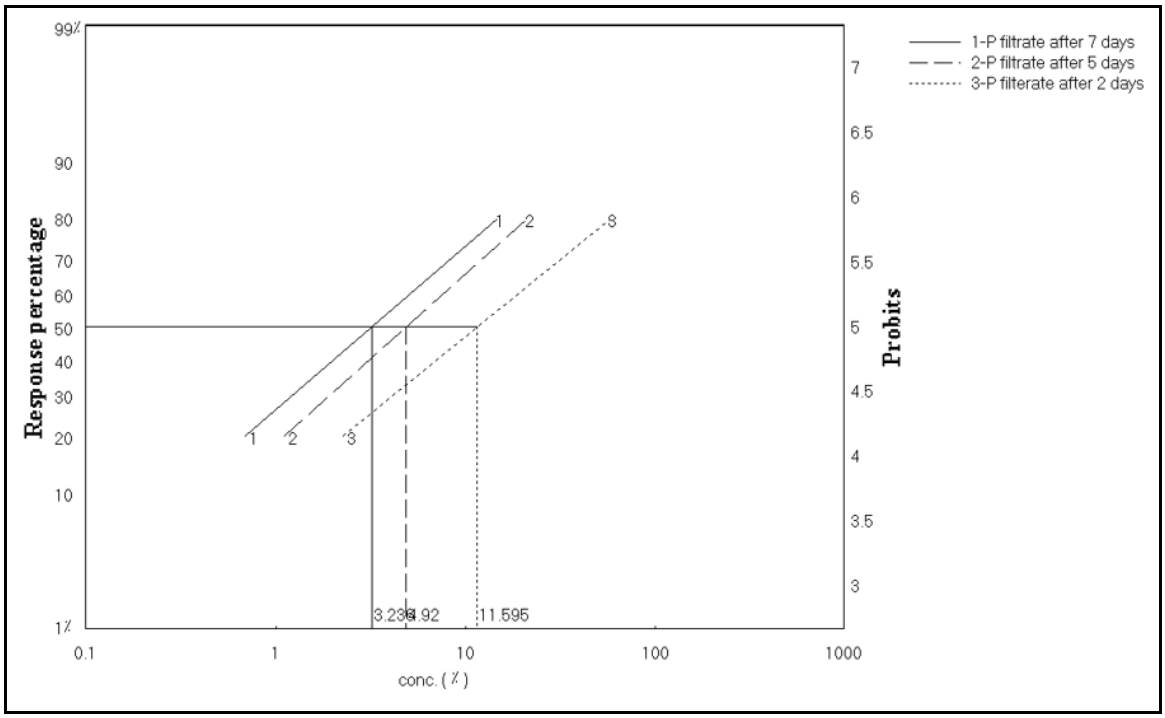

Fig. 3: The mortality percentage and five concentrations of *M. anisopliae* spore suspensions linear relationship after 2, 5 and 7 days.

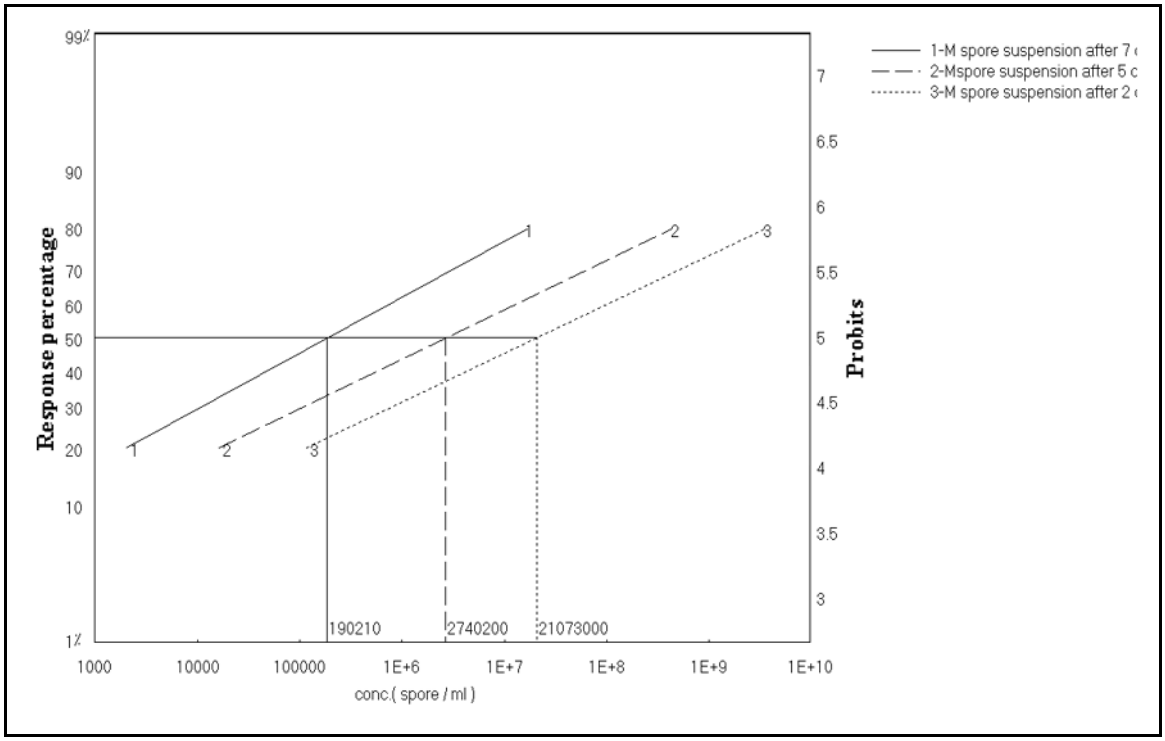

Fig. 4: The mortality percentage and five concentrations of *P. lilacinum* culture filtrates linear relationship after 2, 5 and 7 days

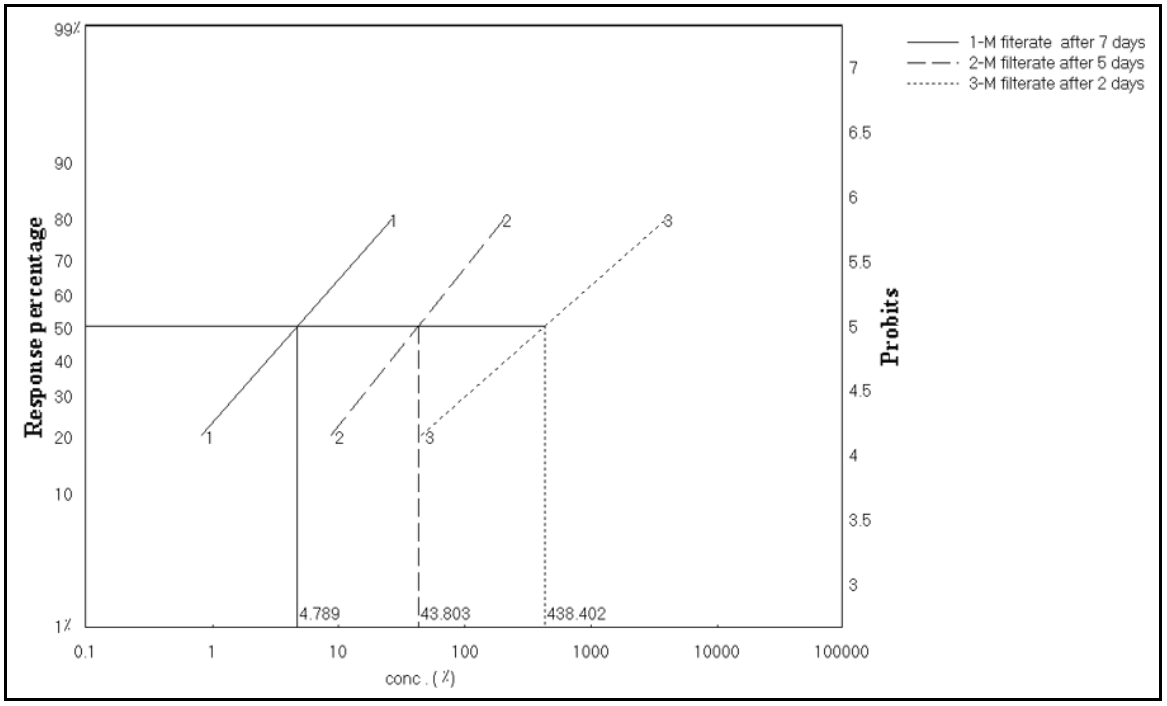

Fig. 5: The mortality percentage and five concentrations of *M. anisopliae* culture filtrates linear relationship after 2, 5 and 7 days.

Histopathological Studies:

Many investigators reported the ability of entomopathogenic fungi in causing severe histological changes and malformation of several insect pests.(Toledo *et al*., 2010) who studied the histopathology caused by entomopathogenic fungi, *B. bassiana* and *M. anisopliae* in the adult plant hopper, *Peregrinus maidis* and noticed that the host whole body was invaded with both spores and hyphae between five and six days post-inoculation, (Farida *et al*., 2018) recorded several histological changes of the 4thinstar larvae of domestic mosquito *Culex pipiens*, which were treated with the entomopathogenic fungi and these histological studies showed many changes and malformation in various parts of the body, especially the cuticle, the adipose cells, and midgut, as well as development and the colonization of the fungus within the tissues. **(**Sanaa *et al*., 2019) revealed histopathological changes of *Galleria mellonella* infected with entomopathogenic fungi include highly destruction of the cuticle and midgut cells. All these results agree with our findings in which light microscopic examination of treated aphid nymph semi-thin sections (Figs. 7 & 8) showed completely disintegration and evanescence of all tissues and cells of whole internal nymph body, there was completely destruction and disappearance of the foregut region, the salivary gland appear shapeless and deformed, in some regions the cuticle appear choppy, discontinuous and disrupted, fungal spores were found scattered in different parts of the nymph body and in some region there were germination of these spores to form fungal mycelium and this suggesting the spread of infection in almost all part of the internal nymph body comparing to untreated nymph body (Fig. 6) in which the body structure appear organized, intact and healthy, the thoracic muscles are evident and proper, the cuticle is clear and can easily distinguish to endo-cuticle and exo-cuticle, the foregut region is obvious divided into normal salivary gland, esophagus and foregut crop.

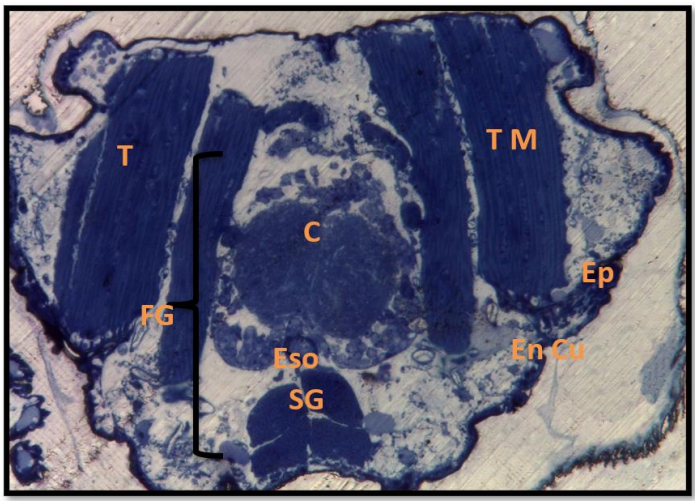

Fig. 6: light microscopic micrograph of semi thin section of untreated Faba bean aphid nymph. TM (Thoracic Muscles), FG (Forgut), C (Crop), Eso (Esophagus), SG (Salivary Gland),EpCu (Epicuticle), EnCu (Endocuticle)

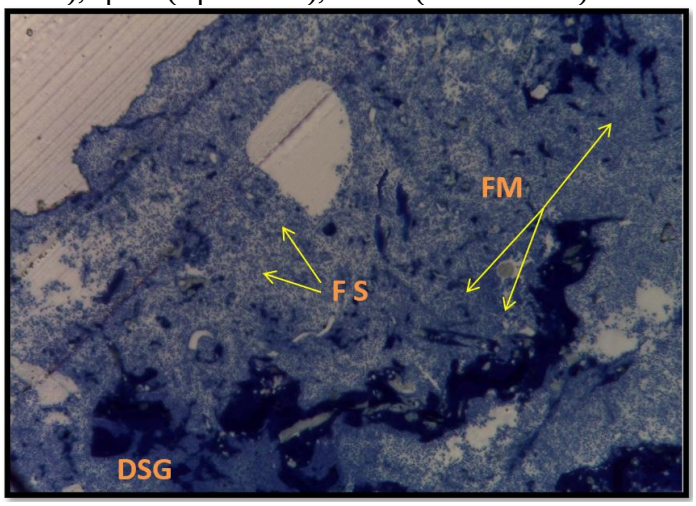

Fig. 7: light microscopic micrograph of semi thin section of treated Faba bean aphid nymph with *M. anisopliae* spore suspension after 7 days post treatment. D S G (Deformated Salivary gland), F S (Fungal Spore), F M (Fungal Mycelium).

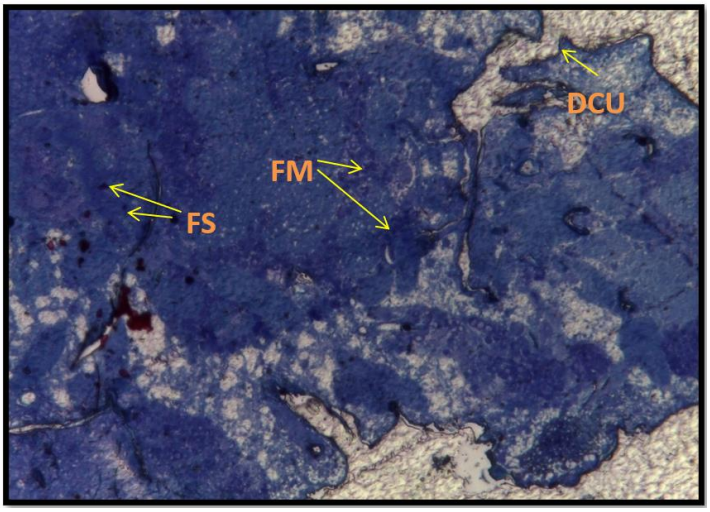

Fig. 8: light microscopic micrograph of semi thin section of treated black aphid bean nymph with *P. lilacinum* spore suspension after 7 days post treatment. DCU (Degenerated Cuticle), F S (Fungal Spore), F M (Fungal Mycelium).

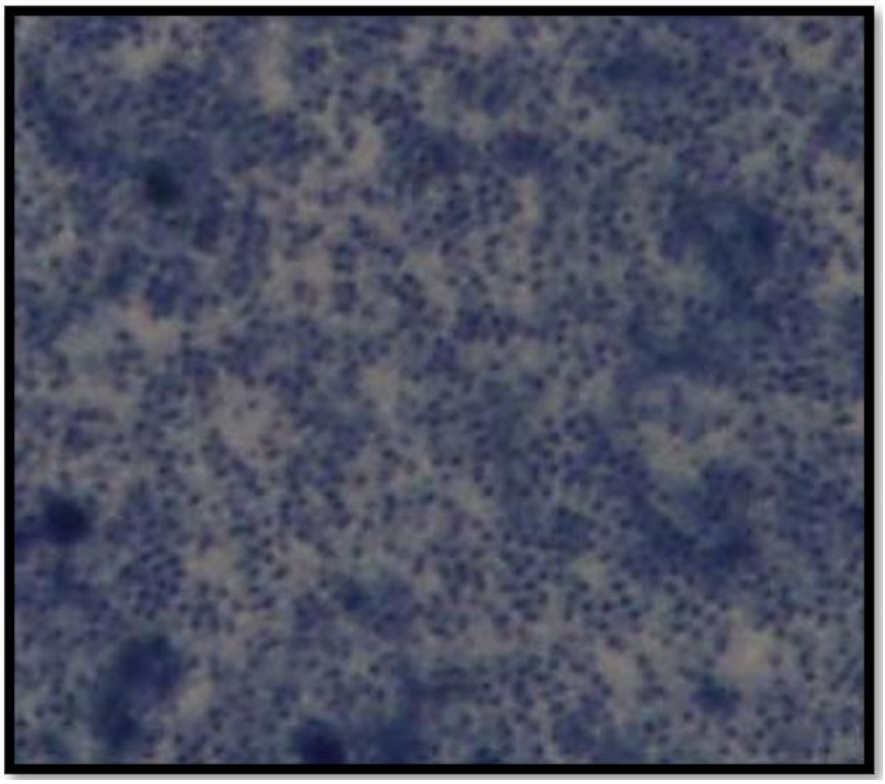

Fig. 9: highly magnified image on some parts showing highly resolution Fungal spores

Ultrastructure Studies:

Transmission electron microscope examination of untreated black bean aphid nymph showed as in light microscope examination normal and organized structure in which the thoracic muscles, mitochondria, tracheae and nucleus appear regular and intact (Fig. 10).

TEM micrographs of treated nymph (Fig. 11) showed complete destruction and disintegration of all internal tissues and cells, (Fig. 11), also there were sever invasion of fungal spores appear attach to all body regions and this was investigated by (Boucias *et al*. 1988). The mode of action of entomopathogenic fungi was summarized in attachment, germination, and penetration of the insect's cuticle then a rapid proliferation of fungal cells inside the host body relates to death of the host.

(Gabarty *et al*., 2014) performed scanning electron microscopy (SEM) examination of *B. bassiana* and *M. anisopliae* infected larvae of the greasy cutworm, *Agrotis ipsilon* (Hufnagel), illustrated adhesion and penetration structures of these fungi. (Sarodee *et al.,* 2016 and Schneider *et al.* 2013) studied the morphological changes in *M. anisopliae* infected *aphis craccivora and Diatraea saccharalis F. (Lepidoptera: Crambid)* respectively and proved that cuticle and abdomen of infected aphid were totally damaged with disfigurement of all the body parts. Also, implied adhesion, growth of the fungus as well as reproduction inside the body of infected aphid.

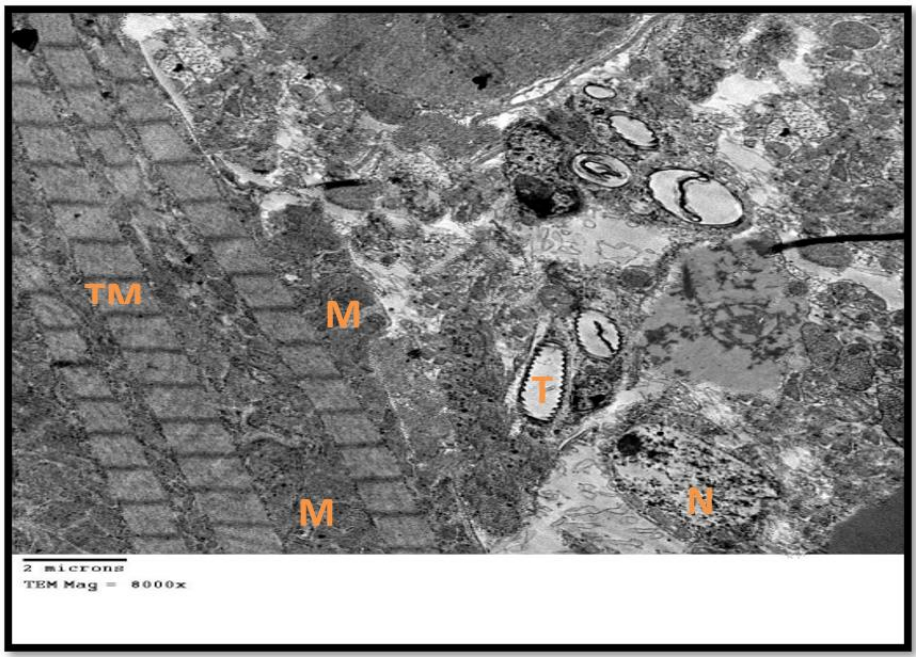

Fig. 10: TEM micrograph of ultra-thin sections of untreated aphid nymph TM: thoracic muscles, M: mitochondria, T: trachea , N : Nucleus

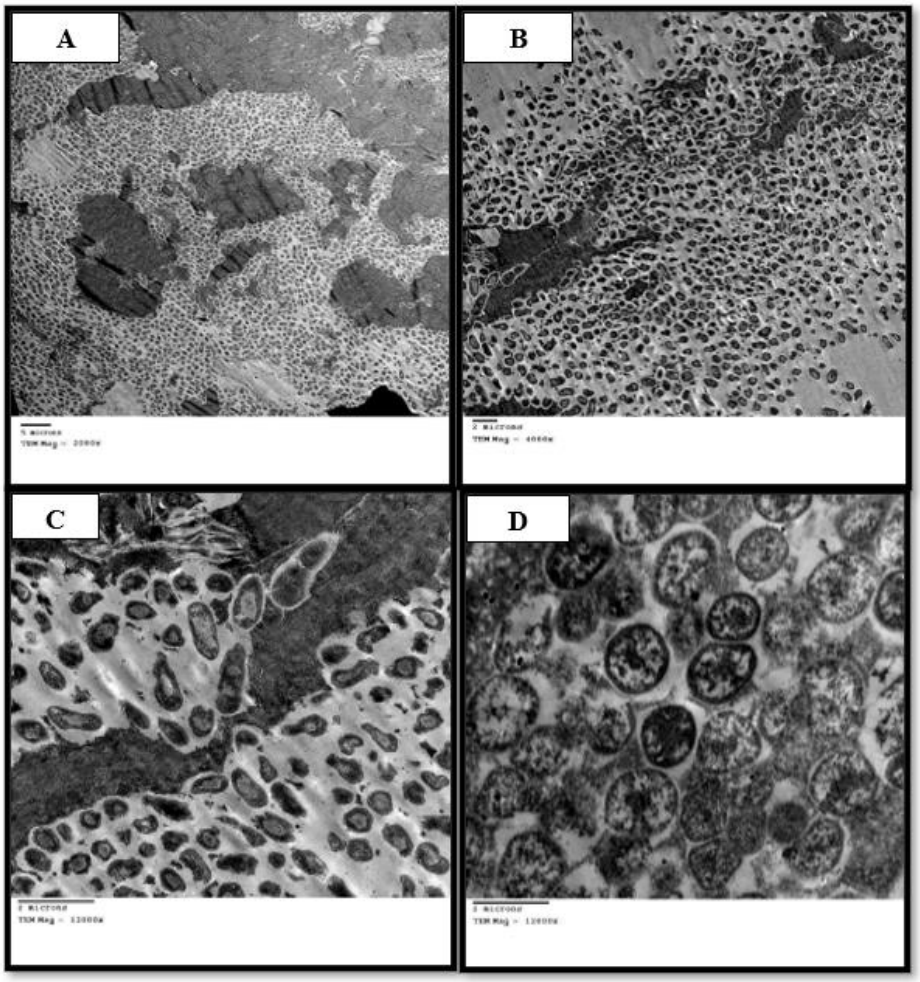

Fig. 11: TEM micrographs of ultra-thin sections of treated aphid nymph with fungal spore suspensions showed a large density of fungal spores scattered inside the nymph tissues. A, B and C: *M. anisopliae* spores D: *P. lilacinum* spores

Conclusion

Black bean aphid *Aphis craccivora* is a pest that causes economic disaster in many crops both directly and indirectly. *Metarihzium anisopliae* and *Purpureocillium lilacinum* proved its efficacy as a biocontrol agent against *A.craccivora* by its toxicological and histological effects which may be caused by the physical invasion of fungal vegetative growth and sporulation or maybe also due to the enzymatic activity or toxin production.

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