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Original article

Virulence of entomopathogenic fungi against *Culex pipiens*: Impact on biomolecules availability and life table parameters

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A B S T R A C T

Culex pipiens mosquitoes considered as vectors for many arboviruses such as the West Nile virus and encephalitis virus showing a global impact on human health. The natural management of the aquatic stages of this pest is crucial for maintaining an insecticide-free and sustained environment. The present work focused on studying the biological and biochemical effects of the entomopathogenic fungi: *Metarhizium anisopliae, Beauveria bassiana*, and *Paecilomyces lilicanus*, against 3rd instar larvae of *Culex pipiens* laboratory colony. The results revealed that *M. anisopliae* showed maximum larval mortality (88%) with the lowest lethal time (LT_{50}) (22.6 hrs) at 10^8 spores/ml followed by *B. bassiana* (73.33%) with LT_{50} (38.35 hrs), while *P. lilicanus* showed minimum percent mortality (65%) with highest LT_{50} (51.5 hrs). The median lethal concentration (LC_{50}) values were found to be 1.027×10^5 spores/ml for *M. anisopliae*, 1.24×10^6 spores/ml for *B. bassiana*, while it was 8.453×10^6 spores/ml for *P. lilicanus*. A reduction in female fecundity, number of hatched eggs, pupation and adult emergence percentage were recorded. The biochemical analysis of the treated larvae revealed different quantitative decrease in total soluble proteins, lipids, and carbohydrate hydrolyzing enzymes compared to control. Histopathological effects of fungal infection upon insect cuticles, muscles, and midgut were investigated. Based on the obtained results, *M. anisopliae* proved its superior virulent effect as a bio-control agent against *Cx. pipiens*.
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1. Introduction

Mosquitoes are dipteran insects acting as biological and mechanical vectors for many parasites and pathogens responsible for communicable diseases. They can spread enzootic or even epizootic diseases such as malaria, dengue fever, and filariasis, etc. World Health Organization (WHO) developed a Global Vector Control Response (GVCR, 2017-2030) to implement vector control strategies that are sustainable (WHO, 2019). Chemical control of vector insects despite being effective, it represents health, environmental and climatic hazard. The relevant situation of insecticide resistance and unsustainable interventions represent challenges in reaching sustainable development goals. All Culicidae are almost bloodsuckers and are responsible for transmitting many important diseases (Medlock et al., 2018). The *Cx. pipiens* acquires most interest because of its large geographical distribution in tropical and sub-tropical countries, which causes a socio-economic impact. Vector control strategies usually target the aquatic stages of mosquitoes in their breeding habitat to counteract the adult resurgence during adult control. Chemical larvicides targeting mosquito breeding sites are responsible for the targeted species' resistance and the long-term secondary effects on non-targeted organisms, harming aquatic fauna (Pintureau, 2009). Developing an alternative strategy for larval control necessitates exploring eco-friendly and biological control methods. Entomopathogenic fungi occupy an immense place among the alternative methods of fighting against insect pests. Application of entomopathogenic fungi in con-

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trolling insect pests gave promising results, i.e., *Beauveria bassiana* (Ziani, 2008) and *Metarhizium anisopliae* (Benserradj and Mihoubi, 2014) to control *Cx. pipiens* for being self-sustaining and efficient alternatives for controlling this pest (Loc et al., 2010). Both species are worldwide containing variable isolates that vary in their host specificity and origin. The application of these fungi will maintain the ecological balance in the surrounding aquatic habitats by controlling the aquatic stages of mosquitoes (Farenhorst and Knols, 2007). The present study describes the efficacy of three entomopathogenic microorganisms against *Cx. pipiens* larvae. The biochemical and histological alterations were observed in different insect tissues.

2. Materials and methods

2.1. Insect colony maintenance

A laboratory strain of *Cx. Pipiens* was obtained from the Research and Training Center on Vectors of Diseases (RTC), Ain Shams University, Cairo, Egypt. The colony was kept in a walk-in chamber insectary at 27 ± 2 °C, 70 ± 10 % RH, and 12:12 h photoperiod. Mosquito larvae were reared in white enamel dishes containing 1500 ml of distilled water. Newly hatched larvae were fed fish food (Tetra-Min, Germany). The adult was reared in wooden cages (24 × 24 × 24 cm) and provided with 10 % sucrose solution and a pigeon for female feeding.

2.2. Fungus culture

Isolates of *Beauveria bassiana* (Balsamo), *Metarhizium anisopliae* (Metschnikoff) Sorokin, and *Paecilomyces lilicanus* (ThomSamson) were obtained from Mycology Center, Faculty of Science, Assiut University, Assiut, Egypt. The isolates were cultured on Sabouraud dextrose yeast agar (SDYA) medium (Sabouraud, 1982) containing 40 g glucose, 20 g peptone, 20 g agar, 2 g yeast extract were dissolved in 1000 ml of distilled water in flasks. The flasks were autoclaved at 121 °C for 15–20 min. Media were poured into Petri dishes and prepared for inoculation (Osman et al., 2020).

2.3. Inoculum preparations

Fungal cultures were plated into the prepared petri dishes, which incubated at 25 ± 2 °C in darkness for 14 days. The conidial suspensions were prepared by scraping cultures with a sterile inoculation needle and transferred to 10 ml of distilled water containing 0.05% Tween 80 in a laminar airflow chamber. The mixture was stirred for 10 min. The hyphal bodies were removed by filtering the mixture through a fine mesh sieve. The conidial concentration of the final suspension was determined by direct count using a hemocytometer (Osman et al., 2020; El-Saadony et al., 2021a).

2.4. Bioassay

The virulence test aimed to compare the efficacy of the three fungal isolates against *Cx. pipiens* 3rd larval instars. Serial dilutions of the fungal spore suspension were prepared in distilled water containing Tween-80 (0.1%) and preserved at 5 °C until used (Alagawany et al., 2021; El-Saadony et al., 2021b,c). The isolates' conidia were tested against larvae by adding the fungal suspensions to plastic cups containing 50 ml of distilled water with 25 larvae of the 3rd instar. Each cup was inoculated with 1 ml of fungal suspensions of 1x10^6, 1x10^7 and 1x10^8 spore/ml. Control treatments were carried out by adding distilled water containing Tween-80 (0.1%) (Haron et al., 2020). Larvae were fed fish food and observed daily. Mortality was recorded at an interval of 24, 48, and 72 h after larval feeding. Mortality percentages were corrected according to Abbotts' formula. Fiducial limits, the median lethal time (LT_{50}), and the median lethal concentration (LC_{50}) values were calculated for each fungal suspension according to Javed et al. (2019).

Regarding the effect of fungal treatment on the mosquito life cycle and female fecundity parameters, the following biological parameters were studied; Mean larval and pupal duration and percentage of pupation. Pupae were sexed and placed in pairs in glass globes. Adult emergence, adult longevity, female fecundity, and egg fertility percentages were calculated (Saad et al., 2021a).

2.5. Effect of entomopathogenic fungi on biomolecules availability in *Cx. pipiens*

2.5.1. Sample preparation

One gram of both untreated and treated larvae was collected 48 h post-treatment and was kept in the freezer (−20 °C) until analysis. Samples were homogenized in distilled water (5 ml/ sample), using a Teflon homogenizer, and centrifuged at 5000 rpm for 20 min at 5 °C. The supernatant was immediately used for the following chemical assay (Saad et al., 2021a).

2.5.2. Estimation of total carbohydrates concentration

Total carbohydrates were estimated by phenol sulfuric acid method according to Saad et al. (2021b). 100 µL phenol (5 g/100 ml) and 200 µL sulfuric acid (conc.) were added to100 µL sample or glucose standard levels. The resulting absorbance (y) was measured at 490 nm after thirty minutes of incubation. The total carbohydrates concentration (x) µg glucose/ mL sample was calculated using the following linear equation, $y = 0.0053x - 0.0193$, $R^2 = 0.9884$.

2.5.3. Estimation of total lipids concentration

Total lipids were estimated quantitatively using phospho-vanillin reagent (20%) prepared by mixing ethanol solution of pure vanillin (0.6% wt./vol.) and concentrated (conc.) phosphoric acid in a ratio of 1:4. The solution was kept in a dark bottle at room temperature (Knight et al., 1972). Briefly, a 250 µL sample was added to 5 ml sulfuric acid and heated in a boiling water bath for 10 min, then 6 ml phospho-vanillin reagent was added. The absorbance of the developed color was read at 525 nm after 45 min. A serial dilution of oleic and palmitic acid mix. (7:3) was used to construct a standard curve (5–25 mg/ml) (Wojciechowska et al., 2019).

2.5.4. Estimation of total protein concentration

Total protein was determined using Coomassie Brilliant Blue (G-250) (Bradford, 1976). Briefly, a solution of Coomassie Brilliant Blue dissolved in 95% ethanol was prepared at a final concentration of 20 mg/ml. Phosphoric acid (85%) was added in a ratio of 1:2, mixture was stirred till the addition of water to a final concentration of (15%,v:v). The filtered solution was kept at 4 °C. 100 µL sample was mixed with 5 ml Bradford reagent for 5 min. Bovine Serum Albumin was used to construct a standard curve for the quantification of total protein in samples. The absorbance was measured at 595 nm (Wojciechowska et al., 2019).

2.5.5. Estimation of carbohydrate hydrolyzing enzymes activity

The hydrolysis activity of trehalose (1.5%), starch (0.5%) and sucrose (2%) with trehalase, amylase and invertase enzymes, respectively was measured at optimum conditions of temperature and pH according to the methods of Ishaaya and Swirski (1976).

2.6. Histopathological studies

The effects of sub-lethal doses (LC_{25}) of the fungal isolates on cells of *Cx. pipiens* larvae were examined using transmission electron microscopy (TEM, JEOL 1000, Japan). Control and treated larvae were prepared for ultrastructural studies (Bowen and Ryder, 1976). Larvae were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (PH 7.2) for an hour followed by an overnight wash in a fresh patch of the same buffer. Specimens were shortly washed in acetate buffer and incubated for an hour at 37 °C in a medium of 5 tablets of P-nitrophenyl phosphate disodium salt, 25 mg lead acetate and 25 ml acetate buffer. The incubation step was stopped by further washing in cacodylate buffer before post fixing in osmium tetroxide followed by routine dehydration and embedding in Araldite. The sections were cut on a Reichert- Jung Ultra-microtome. Semithin and ultrathin sections of 0.5–1.0 μ & 20–60 mm were cut. Semi-thin sections were stained for 1–2 min in toluidine blue stain, washed in tap running water, dried, and mounted in DPX. The ultrathin sections were stained with uranyl acetate and lead citrate stains and then examined microscopically and photographed with TEM (JEOL 1000, Japan) at the Electron Microscope Unit, Mycology Center, Al-Azhar University, Cairo, Egypt.

2.7. Statistical analysis

One way ANOVA at $p \leq 0.05$ was used to analyse the triplicate data means followed by a multiple comparison test (MCT) to indicate the significant differences between means using a statistical analysis system (SAS, 2003).

3. Results

3.1. Virulence of the entomopathogenic fungi against Cx. pipiens

In the present study, three fungal isolates were evaluated according to their virulence against 3rd instar larvae of *Cx. pipiens*. Mortality was recorded at 24, 48 and 72 h. post-treatment.

Data presented in Table 1 showed the daily mortality in the 3rd instar larvae of *Cx. pipiens* upon treatment with three isolates of the entomopathogenic fungi (*M. anisopliae*, *B. bassiana* and *P. lilicanus*). Larval mortality was positively correlated with increasing spore concentration from 10^6 to 10^8 spore/ml in all tested isolates (Fig. 1) (r = 0.9, $p \leq 0.05$). It is worth to mention that *B. bassiana* and *P. lilicanus* cause 50 % mortality in larvae within at least 3 days. In contrast, *M. anisopliae* started to show a 50 % larval mortality within 48 h post-treatment indicating its fast and highly virulent effect.

Regarding the median lethal concentration (LC_{50}), the tested fungal isolates recorded 1.85×10^6 spore/ml, 1.24×10^6 spore/ml, and 8.45×10^6 spore/ml for *M. anisopliae, B. bassiana*, and *P. lilicanus*, respectively 2–3 days post-treatment (Table 1). The data of median lethal time (LT_{50}) revealed that within the applied fungal concentration range (10^6–10^8), *M. anisopliae* required from 22.6 up to 49.2 h to cause 50% larval population mortality (Table 2). Meanwhile, *B. bassiana* and *P. lilicanus* were slower in causing the same effect recording 38.3–75.3 h and 51.6–98.6 h, respectively. *M. anisopliae* has potent effect over the other isolates where having low LC_{50} and LT_{50} values compared to *B. bassiana* and *P. lilicanus*. Only *M. anisopliae* was efficient to exceed 50% larval mortality after 48 h from treatment with about 10^7 spore/ml.

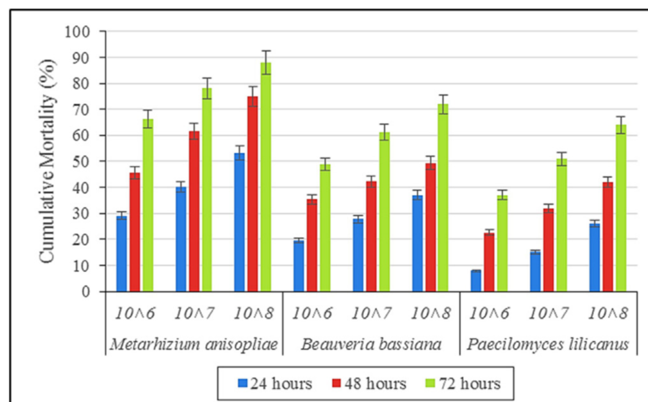

Fig. 1. Cumulative mortality of *Culex pipiens* upon infection with three entomopathogenic fungi at a rate of 10^6, 10^7, and 10^8 spore/ml after 24, 48, and 72 h post exposure.

3.2. Impact of entomopathogenic fungi on the life cycle and reproductive potential:

The impact of the tested fungal species on 3rd instar larvae of *Cx. pipiens* was evaluated on larval and pupal duration, adult emergence, and adult longevity (Table 3). The larval mortality was increased in fungal concentrations dependent manner, it did not significantly affect the larval duration in all fungal treatments. The pupation percentage significantly decreased with increasing fungal spore concentration. *M. anisopliae* severely reduced the pupation process reaching to 27% compared to control (100%). While, *B. bassiana* and *P. lilicanus* reduced the pupation percentage down to 51 and 57 %, respectively. *B. bassiana* significantly increased the pupal duration compared to control. On the other hand, *M. anisopliae* and *P. lilicanus* did not affect the pupal duration. Increasing *M. anisopliae*, *B. bassiana* and *P. lilicanus* spore concentrations significantly decreased the adult emergence down to zero, 52 and 81%, respectively. Regarding adult longevity, *M. anisopliae* did not show a latent effect on adult longevity, the same situation existed for the other two fungi at low concentration. Higher spore concentrations (10^7, 10^8 spore/ ml) from both *B. bassiana* and *P. lilicanus* increased the adult longevity by a magnitude of 1 day. Consequently, the growth index was decreased to half its normal potential from 16.66 down to 7.88 and 6.93 after applying

Table 1
Virulence of fungal isolates at different concentrations against 3rd larval instar of *Culex pipiens* under laboratory conditions.

Fungal Isolate	Time interval (hrs.)	LC_{50} (spore/ml)	Slope value	95% Fiducial limits (lower–upper)
M. anisopliae	24 hrs.	5.604×10^7	0.314 ± 0.0912	1.855–8.15
	48 hrs.	1.85×10^6	0.4017 ± 0.0928	3.39–4.52
	72 hrs.	1.02660×10^5	0.3970 ± 0.1030	1.060–5.19
B. bassiana	24 hrs.	1.53471×10^9	0.2683 ± 0.0958	(1.6×10^8-1.6×10^{16})
	48 hrs.	1.22439×10^8	0.1796 ± 0.0898	($1.8 \times 10^{7-}1.0 \times 10^{38}$)
	72 hrs.	1.23957×10^6	0.3186 ± 0.917	(6.12×10^3-3.92×10^6)
P. lilicanus	24 hrs.	4.61595×10^9	0.3866 ± 0.1122	(4.99–4.82×10^{12})
	48 hrs.	4.63324×10^8	0.2825 ± 0.0938	(8.05×10^7-5.9×10^{11})
	72 hrs.	8.45362×10^6	0.3586 ± 0.0907	(2.75×10^6-2.34×10^7)

Table 2
LT_{50} values of tested fungal isolates at different concentrations against 3rd larval instar of *Culex pipiens*.

Isolates	Concentration (Spore/ml)	LT_{50} (hrs.)	95% Fiducial limits (lower–upper)
M. anisopliae	10^6	49.2172	41.1949–61.0341
	10^7	31.4344	24.3763–37.1782
	10^8	22.6172	15.5580–27.8335
B. bassiana	10^6	75.3511	59.1399–128.3188
	10^7	55.8321	46.9643–71.6969
	10^8	38.3517	29.8666–46.6018
P. lilicanus	10^6	98.5797	77.8924–159.4602
	10^7	74.9221	61.1983–109.4332
	10^8	51.5967	44.1373–62.7252

M. anisopliae and *B. bassiana* at the larval stage compared to control. Meanwhile, *P. lilicanus* treatment reduced the growth potential down to 11.57.

The effect of entomopathogenic fungi on *Cx. pipiens* adult female reproductive potential, also the larval stages in adult females compared to the number of eggs laid per female with control females were showed in Table 4. A significant decrease was recorded in the laid eggs' number after application with *M. anisopliae, B. bassiana*, and *P. lilicanus*. A 2.0 fold decrease in the number of eggs laid /female was recorded upon treatment with 10^8 spore/ml of *M. anisopliae,* meanwhile, *B. bassiana* and *P. lilicanus* fungi caused a reduction in eggs laid per female by a maximum of 1.8 fold upon treatment with 10^7 spore/ml, while a higher concentration of 10^8 spore/ml prevented the egg-laying process to occur.

Fungal treatment showed a slight effect on the egg hatchability, however, monitoring the non-hatched eggs revealed an effect on embryonic development. The majority of the non-hatched eggs (50%) did not contain embryos. Consequently, the sterility index increased by increasing fungal concentration in all strains. *M. anisopliae* and *B. bassiana* increased sterility index up to more than 50% at a fungal spore suspension of 10^7 spore/ml.

3.3. Effect of entomopathogenic fungi on biomolecules availability in Cx. pipiens.

The impact of entomopathogenic fungi on the biomolecules in the insect body; total carbohydrates, proteins, and lipids were quantitatively determined 48 h post-inoculation of *Cx. pipiens* larvae with *M. anisopliae, B. bassiana* or *P. lilicanus* (Fig. 2). This effect may explain the mechanism of nutrient uptake and energy consumption required for infection and fungal growth within its host's body, as well as indicating the virulence in the selected fungal isolates. In the present study, treatment with all fungal isolates resulted in a decline in the total carbohydrates, lipids, and proteins concentrations; however, the depletion was maximum when applying *M. anisopliae* than *B. bassiana* and *P. lilicanus* emphasizing the high virulence of *M. anisopliae* over the other fungi. The magnitude of biomolecules depletion compared to control indicated that the highest decrease in concentration was recorded in lipids (38.2 %), carbohydrates (40.4 %) than proteins (16.6 %) when applying *M. anisopliae*. On the other hand, *B. bassiana* infection decreased the total lipids, carbohydrates, and protien concentrations by 16 %, 15.5 %, and 11%, respectively. Meanwhile, *P. lilicanus* showed minimal effect on biomolecules reduction; total lipids showed a decrease in concentration by 6.7 %, total proteins decreased by 5.78 % and total carbohydrates decreased by 3.5 %.

3.4. Activity of carbohydrate hydrolyzing enzymes

All fungal treatments reduced the secretion of carbohydrates hydrolysis enzymes, i.e., amylase, trehalase, and invertase. The values of these enzymes in untreated *Culex pipiens* 3rd instar larvae accounted for 120, 130, and 202 μg glucose/g. The amylase values in treated larvae with *P. lilicanus, B.bassian*a, and *M. anisopliae* were decreased by 7.2, 22, and 32%, respectively compared to control. In addition, trehalase decremented by 16, 24, and 30% as compared to control. Furthermore, invertase reduced by 8, 20, and 29%, respectively as compared to control. *M. anisopliae* was the most destructive fungi to *Culex pipiens* 3rd instar larvae followed by *B.bassiana* (data not shown). The previous enzymatic activity profile may be attributed to the high virulence of *M. anisopliae* over the other fungal species.

3.5. Histopathological studies

The histopathological effects of the entomopathogenic fungi on *Cx. pipiens* mosquito larvae were examined. Treatment with LC_{25} of fungal isolates revealed an obvious abnormalities and deteriorations in normal ultrastructure of the integument, muscles, and midgut.

3.5.1. The integument

Electro-micrograph of the normal integument in *Cx. pipiens* larvae consisted of an outer epicuticle, lamellated procuticle (exocuticle and endocuticle), and a single layer of the epidermis. The epicuticle is composed of thin non chitinous layer or cuticulin and an amorphous inner epicuticle. The procuticle consists of a series of laminar chitin fibers; each lamina is made up of a sheet of microfibrils that are all oriented in the same direction. The microfibrils of subsequent sheets are positioned at a slight angle to one another. The angle is gradually changing in one direction. Helicoidal structures are examples of such architectures. The epidermis consists of single layer of cells having oval nucleus, which is relatively large, with chromatin scattered around the edges. The plasma membrane is a semipermeable barrier that allows

Table 3
Effect of the fungal isolates on the larval duration, pupation percentage, pupal duration, adult emergence and adult longevity of *Culex pipiens.*

Treatment	Fugal conc. (sp/ml)	Larval duration (Day) Mean ± S.E.	Pupation (%)	Pupal duration (Day) Mean ± S.E.	Adult emergence (%) (A)	Adult longevity (Day) Mean ± S.E. (B)	Growth index (A/B)
Control	**0**	$4.73^{a} \pm 0.15$	100	$1.67^{a} \pm 0.17$	100	$6.00^{a} \pm 0.29$	16.66
M. anisopliae	$\mathbf{10^6}$	$4.83^{a} \pm 0.44$	55	$2.67^{a} \pm 0.44$	76	$6.33^{a} \pm 0.17$	12.00
	$\mathbf{10^7}$	$4.83^{a} \pm 0.44$	37	$3.00^{a} \pm 0.58$	50	$6.33^{a} \pm 0.33$	7.88
	$\mathbf{10^8}$	$5.17^{a} \pm 0.44$	25	$3.00^{a} \pm 0.58$	0	–	–
B. bassiana	$\mathbf{10^6}$	$5.33^{ad} \pm 0.33$	63	$2.17^{bcdf} \pm 0.17$	77	$6.83^{ae} \pm 0.44$	11.27
	$\mathbf{10^7}$	$5.50^{ae} \pm 0.50$	59	$2.33^{be} \pm 0.17$	62	$7.00^{afc} \pm 0.29$	8.85
	$\mathbf{10^8}$	$6.00^{bcde} \pm 0.29$	51	$2.67^{bg} \pm 0.17$	52	$7.50^{bcdef} \pm 0.29$	6.93
P. lilicanus	$\mathbf{10^6}$	$4.83^{a} \pm 0.15$	77	$1.83^{a} \pm 0.17$	94	$6.67^{bcd} \pm 0.17$	14.09
	$\mathbf{10^7}$	$5.00^{a} \pm 0.29$	69	$2.00^{a} \pm 0.29$	89	$6.83^{bd} \pm 0.17$	13.03
	$\mathbf{10^8}$	$5.33^{a} \pm 0.33$	57	$2.00^{a} \pm 0.29$	81	$7.00^{bd} \pm 0.00$	11.57

Means of the same column with the same letters are not significantly different, $p \leq 0.05$. -SE = Standard error.

Table 4
Effect of the fungal isolates on the female reproductive potential of *Culex pipiens*.

Treatment	Conc. (sp/ml)	No. of laid eggs		Hatched eggs		Non-hatched eggs					Sterility Index (%)
		Total No.	Egg/female (Fold change)	Total No.	%	Total No.	With embryo		Without embryo		
							N	%	N	%	
Control	0	3825	225^{a} ± 14.4	3733	97.6	92	76	82.6	16	17.4	0.0
M. anisopliae	10^6	985	140.7^{c} ± 6.7 (-1.6)	838	90.0	84	29	34.5	55	65.5	49.4
	10^7	505	126.2^{c} ± 13.8 (-1.8)	430	87.7	53	21	39.6	32	60.4	55.8
	10^8	110	110^{c} ± 0.0 (-2.0)	94	85.6	16	7	43.7	9	56.3	62.4
B. bassiana	10^6	980	140^{c} ± 11.2 (-1.6)	847	90.4	81	28	34.6	53	65.4	51.4
	10^7	505	126.2^{c} ± 7.5 (-1.8)	444	88	61	24	39.3	37	60.7	57.4
	10^8	–	–	–	–	–	–	–	–	–	–
P. lilicanus	10^6	2150	179.2^{c} ± 14.3 (-1.3)	2003	93.2	147	48	32.6	99	67.4	41.1
	10^7	1260	157.5^{c} ± 9.6 (-1.4)	1144	92.5	116	40	34.5	76	65.5	48.6
	10^8	–	–	–	–	–	–	–	–	–	–

Means of the same column with the same letters are not significantly different, $p \le 0.05$. SE = Standard error, Fold change = No. of eggs laid in control/ No. of eggs laid in treatment.

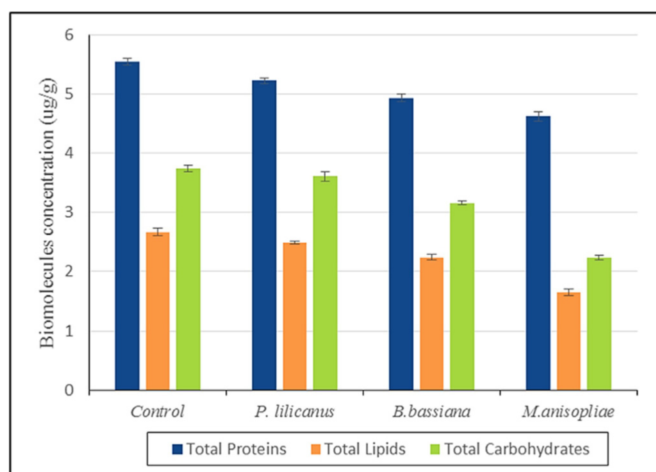

Fig. 2. Effect of entomopathogenic fungi on biomolecules availability in *Cx. pipiens* 3rd instar larvae 48 h post-treatment.

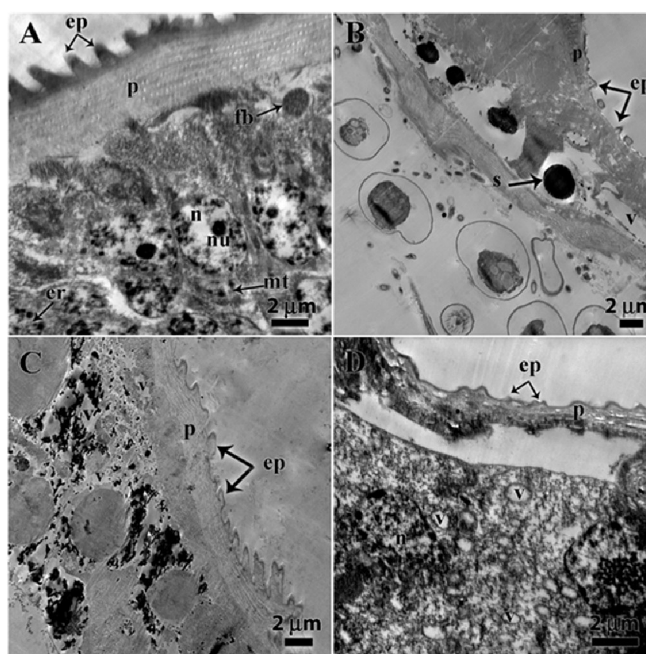

Plate 1. TEM microphotograph of the integument of 3rd instar larva of *Culex pipiens;* A: untreated (x = 8000), B: Treatment with *Beauveria bassiana* (x = 5000), C: Treatment with *Metarhizium anisopliae* (x = 6000), D: Treatment with *Paecilomyces lilicanus* (x = 10.000). ep, epicuticle; p, procuticle; nu, nucleolus; er, endoplasmic reticulum; fb, fat body; mt, mitochondria; n, nucleus; v, vacuole.

molecules and ions to pass between the cytoplasm and the surrounding medium. A few mitochondria can be found dispersed in the cell's cytoplasm. Two membranes combine to form an external limiting membrane that forms the outer shape and an inner membrane that gives rise to the cristae (Plate 1 A). Electro-micrograph of the integument in *Cx. pipiens* treated larvae demonstrate the degradation of the integument and fat body vacuolization. The exocuticle and endocuticle are indistinguishable, and the epidermal cells under the cuticle are blurred. Lysosome leakage can be seen with the adjacent vacuoles, which are responsible for cell lysis. The integument boundary indicates the existence of an exocuticle. The mitochondria's ultrastructure changes show deformation, significant coalescence, and inner damage (Plate 1 B, C, and D). *Beauveria bassiana* infection showed general disorganization in the cuticle resulted in a loss of differentiation in epicuticle and procuticle. The epicuticle showed a discontinuous appearance with loosening in the projections (papillae) bounded to it (Plate 1B). A prominent separation between the epicuticle and the endocuticle was observed. In addition, loss of lamellae in the endocuticle and the appearance of vacuoles in the epidermis. Nuclei were degenerated (pycnotic). Besides, fragmented epidermal cells were noticed. In addition, larvae treated with *Metarhizium anisopliae* showed discontinuation of the epicuticle layer (Plate 1C). The endocuticle layer appeared disorganized and loosed the striated and organized lamellae. Treatment with *Paecilomyces lilicanus* showed that the epicuticle layer became discontinuous and loosed the projections or papillae bounded to it (Plate 1 D). The endocuticle layer appeared disorganized and loosed the striated and organized lamellae. Also, separation of the cuticle layer from the epidermal layer was obvious in addition to the absence of the differentiated layers of the cuticle.

3.5.2. The muscles

The normal skeletal muscles consist of contractile striated fibers lying parallel with one another; each fiber consists of some parallel fibrillae laid in the sarcoplasm. Connective-tissue layer sheaths the longitudinal muscle fibers, the structure of myofibrils shows the presence of thick tubular filaments (myosin) and fine filaments (actin). Regular skeletal muscles are made up of elongated contractile fibers that lie parallel to one another. They are frequently in large numbers. The muscles consisted of striated fibers. Each fiber

consisting of a series of parallel fibrillae or sarcostyles laid down in nucleated plasma or sarcoplasm rich in glycogen. Fibrils are tiny threads with no obvious distinction. The longitudinal muscle fibers are covered by an amorphous layer of connective tissues, which contain many T-tubules. In a detailed manner, the fine structure of myofibrils displays the presence of thick, apparently tubular (presumable myosin) filaments and fine (presumably actin) filaments. The peripheral sarcoplasm of the muscle fibers contains sarcoplasmic reticulum together with small vesicular bodies are observed in those myofibrils. In all these types each sarcostyle or myofibril consists of alternating isotropic and anisotropic segments; these more or less correspond with the pale and dark-staining discs visible in the fixed tissue. In a given fiber, these discs are at approximately the same level in neighboring fibrils, therefore, the entire fiber has a banded or striated appearance. The details of this striation vary in complexity in different muscles. A membrane traverses the light disc, the Z line attached all-round the fiber to the sarcolemma, the compartment between adjacent membranes being termed a sarcomere. In the light disc, on either side of the telophragma, there may be a narrow row of dark dots. The control larvae showed well-organized myofibrils with densely distributed myofilaments (actin and myosin) surrounded by sarcoplasmic reticulum. There are numerous mitochondria and nuclei observed in the sarcoplasm (Plate 2 A). Fungal treatment to *Culex pipiens* 3rd instar larvae revealed a vacuolization and disappearance of the sarcoplasmic reticulum also, shrinkage, reduction, and disorganization in fibrils size was noticed compared to the untreated larvae. The disappearance of mitochondria and destruction of the nucleus with condensed chromatin were observed. Muscles were gradually disorganized (Plate 2 B, C, D). Moreover, *Metarhizium anisopliae* treatment showed the disappearance of the sarcoplasmic reticulum in addition to shrinkage, reduction, and disorganization in fibrils size.

3.5.3. Midgut

The epithelium of the midgut consists of a single layer of columnar cells, which are separated from the hemolymph by a basement membrane, and two layers of visceral muscle fibers. On the basal surface, the plasma membrane is extensively infolded. The lateral cell surfaces were relatively straight in the apical part of the cell; however, towards the basal region, there is an extensive interdigitating between the adjacent cells. Numerous microvilli evaginate from the luminal surface of the epithelial cells. The epithelial cells of the midgut exhibit a uniform structural organization; however, some variations in size, shape, and electron density were observed in the epithelial cells and peritrophic membrane. A single oval nucleus is located towards the apical region of the cell. The cytoplasm contained a well-developed granular endoplasmic reticulum. Mitochondria are generally elongated and are more numerous in the basal region of the cell. The cytoplasm contains many microtubules and microfilaments most noticeably in the apical area of the cell. Multi-vesicular bodies were also frequently observed (Plate 3A).

Ultrastructural changes in epithelial cells of the midgut of treated *Cx. pipiens* larvae revealed lyses of epithelial cells and change in nuclear shape with clumping of chromatin material. The cytoplasm involved cellular vacuolization. Mitochondria and lysosomes degraded. Detachment of peritrophic membrane from the epithelial cells and become malformed. Treatment-induced disappearance of intercellular junction that separates the cell from each other. The endoplasmic reticulum was broken down into separate narrow vascular structures. The cytoplasm of epithelial cells contains multi-vesicular bodies, where the mineralized material is deposited. They occupied a relatively great proportion of the cytoplasmic area (Plate 3 B, C, and D).

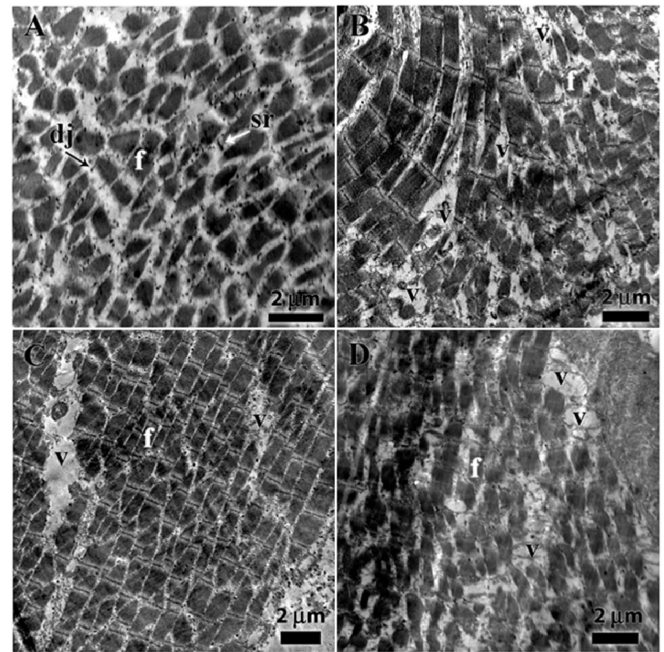

Plate 2. TEM micrograph of cross-sectioned skeletal muscle fibrils in the third instar larva of *Culex pipiens*; A: untreated (x = 20000), B: treatment with *Beauveria bassiana* (x = 10,000), C: treatment with *Metarhizium anisopliae* (x = 5000), D = treatment with *Paecilomyces lilicanus* (x = 10,000). f, myofibrils; sr, sarcoplasmic reticulum; v, vacuole; dj, diad junction.

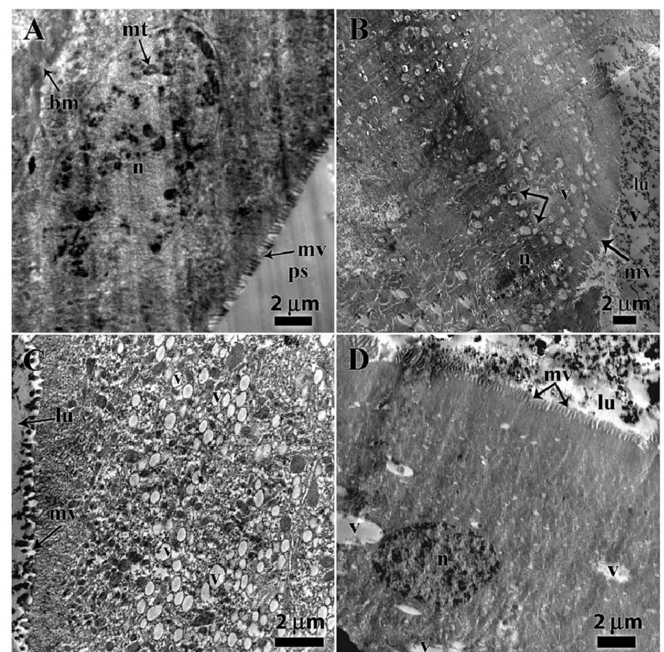

Plate 3. TEM micrograph of midgut in the third instar larva of *Culex pipiens*; A: untreated (x = 8000), B: treatment with *Beauveria bassiana* (x = 4000), C: treatment with *Metarhizium anisopliae* (x = 12000), D = treatment with *Paecilomyces lilicanus* (x = 8000). mv, microvilli; n, nucleus; mt, mitochondria; ps, peritrophic space; bm, basal membrane; lu, lumen.

4. Discussion

Fungi were proposed as effective biocontrol agents against *Cx. pipiens* (Pedrini et al., 2007; Hamid et al., 2013). *M. anisopliae* proved its potent effect over the other two isolates in terms of both having low LC_{50} and LT_{50} values compared to *B. bassiana* and

P. lilicanus. Only *M. anisopliae* was efficient enough to exceed 50% larval mortality after 48 h. from treatment with about 10^7 spores/ml. A comparative virulence study between *B. bassiana* and *M. anisopliae* against *Cx. quinquefasciatus* larvae concluded the same superior effect of the later fungus detected in the present study (LC_{50} of $1.97x10^4$ conidia/ml and LT_{50} of 1 day) (Alves et al., 2002). The same virulence effect was recorded using *B. bassiana* against the mosquito larval stages 1–5 days post-treatment (Hamid et al., 2013). They recorded an increase in larval mortality from 20% (after 24 h) up to 80% (after 96 h from exposure) in older larval stages with LT_{50} of 2.29 h using $0.33x10^7$ spore/ml. Also, *A. aegypti* mosquito larvae were subjected to a controlling strategy using conidia and blastospores of the fungus*, M. brunneum*; the blastospores needed lower LT_{50} values than conidia to attain suppressive effect on larvae, suggesting the former in field application strategies for being more virulent (Alkhaibari et al., 2018).

Also, *M. anisopliae* caused 96% mortality in *Cx. pipiens* after 96 h (Benserradj and Mihoubi, 2014)**.** *M. anisopliae* and *Paecilomyces* spp. Fungi at a rate of 10^8 conidia/ml were tested for their efficacy against *Cx. quinquefasciatus* larvae, caused up to 80 % and 70 % larval mortality, respectively, indicating the promising effect of the former fungus in vector control strategies (Sani et al., 2017).

The potential role of the entomopathogenic fungi as *B. bassiana* and *M. anisopliae* against mosquito adult duration revealed a reduction in adult longevity in the dengue fever mosquito (*Aedes aegypti*) (Darbro et al., 2011). *Cx. pipiens* larval mortality and pupal duration increased upon treatment with fungal suspensions of either *B. bassiana* or *M. anisopliae* also, the percent pupation decreased upon treatment. Adult emergence decreased as a result of both fungal applications (Shoukat et al., 2016).

The effect of entomopathogenic fungi on adult longevity examined in previous studies on *Cx. pipiens* and the beetles, *Anoplophora glabripennis* and *Ostrinia nubilalis* is a reduction in percent pupation, as well as adult longevity were recorded (Abd El-Kareem, 2007; Dubois et al., 2004; Shoukat et al., 2016)**.** The previous plateau in the consumption of the host's biomolecules indicates the dependence of fungi primarily on lipids and carbohydrates than proteins as a source of energy for the fungal infection and growth within the host larvae during the first two days post-infection. The infection process in aquatic stages as in *Cx. pipiens* larvae occur primarily from natural openings as the buccal cavity during feeding then toxins were produced. In this process, larvae were put into dietary stress as conidia are indigestible and hence larvae can't get benefit from ingested food properly (Lacey et al., 1988). There is a new entry to fungi through the siphon tip during the respiration process. *M. anisopliae* with hydrophobic conidia allowing hyphal growth into the tracheal system causing suffocation to the host and eventually its death (Mannino et al., 2019).

The impact of *M. anisopliae* on reducing total proteins, carbohydrates, and lipids was superior to *B. bassiana* upon infecting the green stink bug, *Nezara viridula* emphasizing its pathogenicity (Nada, 2015). Entomopathogenic fungi express an array of genes that are responsible for the nutrient absorption process which is a prerequisite for fungal growth and biomass build-up inside its host (Butt et al., 2016). The role of microbial enzymes involved in the infection and fungal growth process is also crucial. Lipases and protease are from those enzymes that were secreted into the host's body to hydrolyze lipids and proteins in the cuticle, as well as insect hemocoel (Mondal et al., 2016). Infection with virulent fungi results in a depletion of the host's total lipids and proteins. Microbial lipase was produced in *M. anisopliae* at an earlier stage than that of *B. bassiana*, which was produced only at the stationary phase (Mondal et al., 2016). Hence, the depletion of the hosts' lipids is faster upon infection with *M. anisopliae* than *B. bassiana* infection.

Carbohydrates constitute the main source of glucose essential for variable biological processes in the insect body. Enzymes that are involved in carbohydrate metabolism through hydrolysis reactions are amylase, trehalase, and invertase.

Trehalase enzyme is responsible for the hydrolysis of trehalose, a disaccharide that is considered the blood sugar in many insects (Thompson, 2003). Trehalose is also considered an important energy source for entomopathogenic fungi growth, hence, there is a correlation between trehalose concentration, trehalase activity, and pathogenicity (Zhao et al., 2006).

Moreover, the activity of trehalase and invertase enzymes decreased in the green stink bug, *N. viridula* upon infection with *M. anisopliae* and *B. bassiana* (Nada, 2015). Also, *M. anisopliae* infection to *Locusta migratoria* resulted in a depletion of trehalose activity. in host hemolymph accompanied by an elevation in trehalase enzyme activity, probably a fungal trehalase (Zhao et al., 2007). Trehalase activity was increased in field strains of *S. littoralis* in response to spinetoram treatments accompanied with altered carbohydrates metabolism, releasing the stored energy source is an indicator of biological stress (Fahmy and Dahi, 2009). General reduction activity of amylase, invertase, and trehalase was recorded in the American bollworm larvae (*Helicoverpa armigera*) and Cotton aphid adults treated with fungal bio-insecticide (Al-Shannaf et al., 2012; Khaleil et al., 2016). The same effect of spinosad bioinsecticide on reducing carbohydrate hydrolyzing enzyme activity was recorded in *S. littoralis*, *Pectinophora. gossypiella* and *Earias insulana* larvae (Aumar et al., 2006; HALA et al., 2008)**.**

Anopheline and culicine mosquitoes when subjected to stress by bio-insecticides, they expressed decreasing carbohydrate, lipids, and protein concentrations; an explanation for this decrease could be attributed to blocking of the alimentary canal by the entomopathogen leading to a decrease of total ingested food affecting carbohydrate concentration (Sharma et al., 2011). Under stress, energy production is mainly through lipid catabolism leading to a decline in lipid concentration (Sharma et al., 2011). As for protein concentration, there may be a decrease in the protein expression process by the action of the produced toxins. However, the role of fungal toxins in biomolecules availability should be studied at the molecular level to unveil the probable inhibitory effect on the gene expression process.

Histopathological effects of entomopathogenic fungi on various insect structures were investigated (Abdel-Gawad et al., 2020; Gabarty et al., 2014). The relevant site of fungal attach is the insects' integument. It is the first and outermost protective tool against mechanical, physical, chemical, and biological damage (Wigglesworth, 1972). Entomopathogenic fungi exert their effect on the insect cuticle both mechanically (through the penetration effect), as well as through chemical lysis of the cuticle and the whole body tissues by the action of chitinase, protease, and lipase enzymes (Benserradj and Mihoubi, 2014; Mondal et al., 2016).

The normal cuticle in non-treated larvae is differentiated into outer exocuticle and inner endocuticle. In the present study, infection of *Culex pipiens* 3rd instar larvae by *M. anisopliae, B. bassiana,* or *P. lilicanus* entomopathogenic fungi resulted in a deteriorating effect on the insect cuticle. The effect was expressed as non-differentiation of the exocuticle and endocuticle, the same effect was observed by the action of *B. bassiana* (Farida et al., 2018)*.* The ultrastructural damaging effect of fungal infection in *Culex pipiens* larvae was extended to internal tissues including muscles, intestine, and adipose tissue (Ali and Abdallaa, 2012). The present study revealed the appearance of vacuoles because of depletion in tissues and cell organelles by either the action of degrading enzymes or the mechanical destructive effect of fungal spore (Bawin et al., 2016; Farag et al., 2021)**.** Histological changes in the alimentary canal accompanied by destruction in the brush border were also observed in *Cx. quinquefasciatus* and *Aedes aegypti*

upon infection with indigenous fungi (Ragavendran et al., 2019). A collapse and hyperplasia in mosquito gut epithelia were also observed with degeneration in nuclei (Ragavendran et al., 2017).

5. Conclusion

Entomopathogenic fungi are considered a naturally occurring microbial control agent against many insects, playing a role in decreasing the host population in epizootics. Most of them start the infection process via the gut. The cuticle penetration is assisted by fungal enzymes, in addition, the toxin production induced the host's immune response, including activation/deactivation of host enzymes. The fungal propagation within-host body leading to a remarkable depletion of host biomolecules availability affecting variable parameters in the host life cycle and finally host death in a susceptible host. The difference in cuticular structure and epicuticle chemical composition is a factor that determines the host-fungus relationship. The variation between the selected fungal isolates in toxicity, effect on host biomolecules availability, and histopathological effects may be attributed to these factors. In conclusion, it is recommended to implement the entomopathogenic fungus, *M. anisopliae,* in vector control and management programs as a virulent, safe, self-propagating, and ecologically compatible microbial control agent.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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