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

Heba M. Hamama a, Ola H. Zyaan b, Ola A. Abu Ali c, Dalia I. Saleh c, Hend A. Elakkad d, Mohamed T. El-Saadony e,⇑, Shaimaa M. Farag b

a Entomology Department, Faculty of Science, Cairo University, Cairo, Egypt
b Entomology Department, Faculty of Science, Ain Shams University, Cairo, Egypt
c Department of Chemistry, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia
d Biochemistry Department, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt
e Department of Agricultural Microbiology, Faculty of Agriculture, Zagazig University, Zagazig 44511, Egypt

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-
trolling insect pests gave promising results, i.e., Beauveria bassiana (Ziani, 2008) and Metarhizium anisopliae (Benserrad 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. P. 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 adults were 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 lilacinus (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 Abbott’s formula. Fiducial limits, the median lethal time (LT₅₀), and the median lethal concentration (LC₅₀) 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, \[ y = 0.0053x - 0.0193, \text{R}^2 = 0.9884. \]

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 phosho-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 trehalase (1.5%), starch (0.5%) and sucrose (2%) with trehalase, amylose 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 then washed in acetic acid 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 buffer and incubated for an hour at 37 °C. The sections were then washed 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 & 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 ≤ 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 C. 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 ≤ 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 to 10^6 spore/ml, 1.24 to 10^6 spore/ml, and 8.45 to 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.

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

| 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 × 10^7      | 0.4017 ± 0.0928 | 3.39–4.52                      |
|               | 72 hrs.             | 1.0260 × 10^7    | 0.3970 ± 0.1030 | 1.060–5.19                     |
| B. bassiana   | 24 hrs.             | 1.53471 × 10^8   | 0.2683 ± 0.0958 | (1.6 × 10^8–1.6 × 10^8)        |
|               | 48 hrs.             | 1.22439 × 10^8   | 0.1796 ± 0.0898 | (1.8 × 10^8–1.0 × 10^8)        |
|               | 72 hrs.             | 1.23957 × 10^8   | 0.3186 ± 0.917  | (6.12 × 10^6–3.92 × 10^6)      |
| P. lilicanus  | 24 hrs.             | 4.61595 × 10^6   | 0.3860 ± 0.1122 | (4.92–4.82 × 10^6)             |
|               | 48 hrs.             | 4.63314 × 10^6   | 0.2825 ± 0.0938 | (3.05 × 10^6–5.9 × 10^6)       |
|               | 72 hrs.             | 8.45362 × 10^6   | 0.3586 ± 0.0907 | (2.75 × 10^6–2.34 × 10^6)      |
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 per 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 protein 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 piperis 3rd instar larvae accounted for 120, 130, and 202 µg glucose/g. The amylase values in treated larvae with P. lilicanus, B.bassiana, 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 piperis 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...
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 1A). 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 loosened 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

| 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° ± 14.4 | 3733 | 97.6 | 92 | 76 | 82.6 | 16 | 17.4 | 0.0 |
| M. anisopliae | 10⁶ | 985 | 140.7° ± 6.7 (-1.6) | 838 | 90.0 | 84 | 29 | 34.5 | 55 | 65.5 | 49.4 |
| | 10⁷ | 505 | 126.2° ± 13.8 (-1.8) | 430 | 87.7 | 53 | 21 | 39.6 | 32 | 60.4 | 55.8 |
| B. bassiana | 10⁶ | 110 | 11° ± 0.0 (-2.0) | 94 | 85.6 | 16 | 7 | 43.7 | 9 | 56.3 | 62.4 |
| | 10⁷ | 980 | 140° ± 11.2 (-1.6) | 847 | 90.4 | 81 | 28 | 34.6 | 53 | 65.4 | 51.4 |
| | 10⁸ | 505 | 126.2° ± 7.5 (-1.8) | 444 | 88 | 61 | 24 | 39.3 | 37 | 60.7 | 57.4 |
| P. lilicanus | 10⁷ | 2150 | 179.2° ± 14.3 (-1.3) | 2003 | 93.2 | 147 | 48 | 32.6 | 99 | 67.4 | 41.1 |
| | 10⁸ | 1260 | 157.5° ± 9.6 (-1.4) | 1144 | 92.5 | 116 | 40 | 34.5 | 76 | 65.5 | 48.6 |

Means of the same column with the same letters are not significantly different, $p < 0.05$. 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, nucleus; er, endoplasmic reticulum; fb, fat body; mt, mitochondria; n, nucleus; v, vacuole.
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 (presumably 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 sarcosome. 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 3 A).

Ultrastructural changes in epithelial cells of the midgut of treated Cx. pipiens larvae revealed lysed 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).

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 LC50 and LT50 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 (LC50 of 1.97x10^4 conidia/ml and LT50 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 LT50 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 LT50 values than conidia to attain suppressive effect on larvae, suggesting the former in field application strategies for being more virulent (Alkhailbari et al., 2018).

Also, M. anisopliae caused 96% mortality in Cx. piperi after 96 h (Benserradj and Mihoubi, 2014). M. anisopliae and Paecilomyces spp. Fungi at a rate of 10^6 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. piperi 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. piperi and the beetles, Anoplophora glabrripennis 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. piperi 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 hemocoeel (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 host’s 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 trehalase 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-Shanaaf et al., 2012; Khaleel et al., 2016). The same effect of spinosad bioinsecticide on reducing carbohydrate hydrolyzing enzyme activity was recorded in S. littoralis, Pectinophora gossypiella, and Eutrus insulana larvae (Aumar et al., 2006; HALA et al., 2008).

Anopheleline 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 epicuticle and inner endocuticle. In the present study, infection of Culex piperi 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 piperi larvae was extended to internal tissues including muscles, intestine, and adipose tissue (Ali and Abdalla, 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 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.

Acknowledgements

Taif University Researchers Supporting Project number (TURSP-2020/220), Taif University, Taif, Saudi Arabia.

References

Abd El-Kareem, S., 2007. Biological and histopathological studies on the effect of certain entomopathogenic microorganisms on the European corn borer, *Ostrinia nubilalis* Hübnér (Lepidoptera: Pyralidae). M. Sc. Thesis, Fac. Sci., Ain Shams Univ. Abdel-Gawad, A.M., Khalifa, M.M., Elhariri, M.D., Mahmoud, M.A., El-Geneady, M.A., 2020. Histopathological and scanning electron microscope studies of entomopathogenic fungus (beauveria spp.) on different stages of Musca domestica (diptera: Muscidae). J. Egypt. Soc. Parasitol. 50, 105-112. Al-Shanafi, H., Mead, H.M., Sabry, A., 2012. Toxic and biochemical effects of some bioinsecticides and igs on american bellworm, Helocoverpa armigera (Hüb.) (Noctuidae: Lepidoptera) in cotton fields. J. Biofertil. Biopestic. 3 (118). Journal, B.S., 2012. Histological study of Culex pipiens pipiens larvae and adults infected with Beauveria bassiana. Baghdad Sci. J. 9 (2), 187–193. Alagawany, M., Madlouf, M., El-Saadony, M.T., Reda, F.M., 2021. *Paenibacillus polymyxus* (LM31) as a new feed additive: Antioxidant and antimicrobial effect of some compounds on carbohydrate hydrolyzing enzymes of cotton leafworm, *Spodoptera littoralis* (Boisd.), Egypt. J. Agric. Res. 86 (6), 2169–2179. Hamada, M., Hala, M., El-Sheakh, A., Soliman, B., Desuky, W., Abo-Ghalia, A., 2008. Biochemical effect of some compounds on carbohydrate hydrolyzing enzymes of *Oreochromis niloticus* with Beauveria brongniartii cultures against the Asian longhorned beetle, *Anoplophora glabripennis* (Coleoptera: Cerambycidae). Biol. Control. 31 (3), 320–326. El-Mouaghith, Abdu, Hashem, Halim and Lokma Noha, 2016. Biocontrol effect of some compounds on carbohydrate hydrolyzing enzymes of *Anoplophora glabripennis* (Coleoptera: Cerambycidae). Biol. Control. 31 (3), 320–326. El-Saadony, M.T., Alkharthi, F.M., Alzahrani, S.O., Shafi, M.E., El, Abdel-Hamid, S., Taha, T.F., Aboelelnin, S.M., Soliman, M.A., Ahmed, N.H., 2021a. Impact of mycogenic zinc nanoparticles on performance, behavior, immune response, and microbial load in Ochrochromis niloticus. Saudi J. Biol. Sci. 28 (8), 4592–4604. El-Saadony, M.T., Saad, A.M., Najjar, A.A., Alzahrani, S.O., Alkharthi, F.M., Shafi, M.E., Selem, E., Desoky, E.-S.-M., Fouda, S.-E.-S.-E.-S., El-Tahan, A.M., 2021b. The use of biological selenium nanoparticles in controlling *Trichicum aestivum* L. crown root rot under different wheat species and improve yield under drought and heat stress. Saudi J. Biol. Sci. 28 (8), 4461–4471. El-Saadony, M.T., Sitohy, M.Z., Ramadan, M.F., Saad, A.M., 2021c. Green nanotechnology for preserving and enriching yogurt with biologically available iron (II). Innovative Food Science & Emerging Technologies 69, 102645. Fahmy, N., Dahi, H., 2009. Changes in detoxifying enzymes and carbohydrate metabolism associated with spinetorin in two field-collected strains of Spodoptera littoralis (Lep.). Egypt. J. Biol. Chem. Taxonomy & Pest Control 1 (1), 17–26. Farag, S., Hussein, A.M., Hafez, E.S., Khalel, S.A., Islam, M.O., Zyaan, H.O., 2021. Entomopathogenic, biological, and histopathological alterations induced by pomegranate peel extract, Punica granatum against Culex pipiens L.(Diptera: Culicidae). Egypt. J. Aquat. Fish. Biol. 25, 139–161. Farenhorst, M., Knols, B.G., 2007. Fungal entomopathogens for the control of adult mosquitoes: a look at the issues, Proceedings of the Netherlands Entomological Meeting, pp. 51–59. Farida, B., Sonia, H., Hakima, M.-K., Fatma, B., Fatma, H., 2018. Histological changes in the larvae of the domestic mosquito Culex pipiens treated with the entomopathogenic fungus Beauveria bassiana. Sci. Res. Essays 13, 1-10. Gabarty, A., Salem, H.M., Fouda, M.A., Abas, A.A., Ibrahim, A.A., 2014. Pathogenicity induced by the entomopathogenic fungi Beauveria bassiana and *Metarhizium anisopliae* in *Agrotisipson* (Hufn.). J. Radiat. Res. Appl. Sci. 7 (1), 95–100. Halal, M., El-Shekh, A., Sood, B., Desuky, W., Abo-Chalia, A., 2008. Biochemical effect of some compounds on carbohydrate hydrolyzing enzymes of *Anoplophora glabripennis*, *Spodoptera littoralis* (Boisd.), Egypt. J. Agric. Res. 86 (6), 2169–2179. Hamed, S., Malouane, F., Bissaad, F.Z., Benzaïma, F., 2013. Study about the effect of Beauveria bassiana (Vuillemien IN 1912) on the aquatic stages of Culex pipiens (LINNÉ, 1758). Haron, E., Ahmed, M., Ali, S., Abas, A., Elshair, E., 2020. Evaluate the effects of entomopathogenic fungus on Wheat Aphid, *Schyzaphis graminum* (Rondani)(Hemiptera: Aphididae). Egypt J. Biofertil. Biopestic. 21 (9), 1–17. Khaled, M., El- Mouaghith, Abdu, Hashem, Halim and Lokma Noha, 2016. Biocontrol potential of entomopathogenic fungus, Trichoderma hamatum against the cotton aphid, *Aphis gossypii* GOODEY. JOSR Environ. Sci. Toxicol. Food Technol. (JOSR- ETFT). 10 (5), 11–20. Knight, J.A., Anderson, S., Rawle, J.M., 1972. Chemical basis of the suflo-phospho-vanillin reaction for estimating total serum lipids. Clin. Chem. 18, 199-202. Lacey, Cynthia M., Lacey, Lawrence A., Roberts, Donald R., 1988. Route of invasion and pathogenicity of *Metarhizium anisopliae* in Culex quinquefasciatus. J. Invertebr. Pathol. 52 (2), 108–118. Loc, N.T., Chi, V.T.B., Nhan, N.T., Hong, T.T.B., Chi, N.T.P., Nghia, N.T., 2010. Exploitation of *Beauveria bassiana* and *Metarhizium anisopliae* as potential biocontrol agents in integrated mosquito control (pmg) on citrus. Online 17, 152–163. Mannino, M., Constanza, Huarte-Bonnet, Carla, Davyt-Colo, Belén, Pedrini, Nicolás, 2019. Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. J. Fungi 5 (2), 33. https://doi.org/10.3390/jof5020033. Medlock, J., Balenghien, T., Atten, B., Versteirt, V., Schaffner, F., 2018. Field sampling methods for mosquitoes, sandflies, biting midges and ticks: VectorNet project 2014-2018. EFSA Supporting Publications 15 (6), 1435E.
Mondal, Subhoshmita, Baksi, Shibashish, Koris, Andras, Vatai, Gyula, 2016. Journey of enzymes in entomopathogenic fungi. Pacific Sci. Rev. A: Nat Sci. Eng. 18 (2), 85–99.

Nada, Maha, 2015. Response of green stinkbug Nezara viridula (Linnaeus), to the activity of entomopathogenic fungi Beauveria bassiana and Metarhizium anisopliae. J. Plant Prot. Pathol. 6 (12), 1633–1644.

Osman, M., Sigloho, C., Mueller, M.J., Waller, F., 2020. An improved growth medium for enhanced inoculum production of the plant growth-promoting fungus Serendipita indica. Plant methods 16, 1–7.

Pedrini, Nicolás, Crespo, Rosana, Juárez, M. Patricia, 2007. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comp Biochem. Physiol. Part – C: Toxicol. Pharmacol. 146 (1-2), 124–137.

Pintureau, B., 2009. Application to an arthropod pests and to weeds. Ed. Elipsess, Paris, 128.

Ragavendran, C., Manigandan, V., Kamaraj, C., Balasubramani, G., Prakash, J.S., Perumal, P., Natarajan, D., 2019. Larvicidal, histopathological, antibacterial activity of indigenous fungus Penicillium sp. against Aedes aegypti and Culex quinquefasciatus (Say) (Diptera: Culicidae) and its acetycholinesterase inhibition and toxicity assessment of zebrafish (Danio rerio). Front. Microbiol. 10, 427.

Ragavendran, C., Mariappan, T., Natarajan, D., 2017. Larvicidal, histopathological efficacy of Penicillium daleae against larvae of Culex quinquefasciatus and Aedes aegypti plus biotoxicity on Artemia nauplii a non-target aquatic organism. Front. Pharmacol. 8, 773.

Saad, A.M., El-Saadony, M.T., El-Tahan, A.M., Sayed, S., Moustafa, M.A., Taha, A.E., Taha, T.F., Ramadan, M.M., 2021. Polyphenolic extracts from pomegranate and watermelon wastes as substrate to fabricate sustainable silver nanoparticles with larvicidal effect against Spodoptera littoralis. Saudi J. Biol. Sci. 28(10), 5674–5683.

Zhao, H., Wang, Z.-K., Yin, Y.-P., Li, Y.-L., Li, Z.-L., Peng, G., Xia, Y., 2007. Trehalose and trehalose-hydrolyzing enzyme in the haemolymph of Locusta migratoria infected with Metarhizium anisopliae strain CQMa102. Insect Sci. 14 (4), 277–282.

Ziani, J., 2008. Application de Beauveria bassiana contre la punaise terne Lygus lineolaris (Palisot de Beauvois) (Hémiptères: Miridés) dans les vignobles. Thesis.