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The Biochemical Effects of *Beauveria bassiana* and *Metarhizium anisopliae* on 3rd instar Larvae of *Culex pipiens* L. (Diptera: Culicidae)

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**ARTICLE INFO ABSTRACT**

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**Keywords:**
*Culex pipiens*; *Beauveria bassiana*; *Metarhizium anisopliae*; Biopesticides; Total protein; SDS-PAGE.

The biochemical effects of two entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, were studied in the 3rd instar larvae of *Culex pipiens* in the laboratory. Results revealed significant reduction in the total proteins of the treated larvae compared to the control larvae. The biochemical studies using (SDS-PAGE) revealed that there was a reduction in the number of protein bands due to the treatment with either of the two fungi. The obtained results indicated that the application of entomofungi as larvicidal agents against mosquito larvae caused significant changes in the total protein profile of *Cx. pipiens* larvae suggesting that toxins secreted by these pathogens caused damage to the larval proteins which finally leads to larval death. The conclusion is that the fungal pathogens are important as natural biological control agents of many insect and other arthropods and frequently cause epizootics that significantly reduce host population. This study recommends the use of *B. bassiana* and *M. anisopliae* as biological control agents to the control of *Cx. pipiens* larvae in order to suppress this medical vector for public health.

**INTRODUCTION**

Mosquitoes (Diptera: Culicidae) species are vectors responsible for the transmission of several infectious diseases with medical and veterinary importance including filariasis, malaria, and arboviruses (Goddard 2008; Mullen & Durden 2009; Medlock et al. 2012). Risk for human infection considerably enlarged during the last decades due to climate changes and increasing global trade (Reiter 2001; Medlock et al. 2012; Boukraa et al. 2013). *Culex pipiens* is one of the most annoying vectors of pathogens for humans. The best counteractive action of mosquito borne diseases is accomplished by decreasing the mosquito population in any of the different life cycle stages, for example, using larvicidal substances. Currently, some problems are caused by the multiple usages of chemical insecticides and reported with respect to the persistence and increase of non-biodegradable chemicals in the environment, the biological enlargement through the food chain, the toxic effect to human health and to non-target organisms, and the increase of insecticide resistance (Rawani et al. 2009).

Integrated pest management is now encouraged due to harmful side effects of
the chemical insecticides classically used for mosquito control and insect resistance
development (Nauen 2007; Rattner 2009; Rivero et al. 2010).

*Beauveria bassiana* and *Metarhizium anisopliae* fungus strain is highly
specific biopesticide to Mosquitoes and is considered safe to non-target invertebrates.

*B. bassiana* and *M. anisopliae* have been extensively studied due to their
simple life-cycle and thereby easy production of steady aerial spores which are the
infectious propagules (Scholte *et al.* 2004; Kanzok & Jacobs-Lorena 2006; Seye *et
al.* 2013). The infection process starts by contact of the spores to the host cuticle.
Sometimes, conidium attaches to the cuticle or secretes mucus for adhesion during its
germination and swelling (Hajek and St. Leger, 1994). Some structures and broad
process are involved in the penetration of the host cuticle and the mechanism of
different fungus may also vary. After the penetration through the cuticle and insect
epidermis, the fungus multiplies into the body cavity of insect and also more fungi
are adapted to aquatic environments or simply ingested by larvae. Some ingested
spores may mechanically block the mouth parts while others can attach inside the
digestive tract (Federici, 1981; Butt *et al.* 2013).

The aim of this study was to evaluate the biochemical changes in the 3rd instar
larvae of *Cx. pipiens* after exposure to biopesticide fungi *B. bassiana* and *M.
anisopliae* under laboratory conditions

**MATERIALS AND METHODS**

**Insect culture:**

Mosquitoes were maintained in insectaries under controlled laboratory
conditions of temperature (27±2°C), relative humidity 70-80%, and light –dark
period (8-16 hrs).

Larvae were reared in white enamel pan containing 1.5 liter of de-chlorinated
tap water. Larvae were provided with tetra-amine (tropical fish food) sprinkled twice
daily over the water surface of breeding pans. The breeding water was slightly
aerated with a small air pump for about 5 minutes every day. Developed pupa were
transferred daily to plastic cup containing de-chlorinated tap water then moved into
the breeding screened wooden cages (30*30*30 cm³).

Emerged adults were fed on 10% sucrose solution. Adults were fed on blood
after three days to lay egg-batches which moved to the white enamel pans containing
de-chlorinated tap water for hatching. When mosquito larvae developed to the 2nd
instar, they were poured into clean pans and observed daily. Early third larval instars
were treated by the LC₅₀ for each fungus according to the method described by

**Fungi source:**

Entomopathogenic fungus, *Metarhizium anisopliae* and *Beauveria bassiana*
was procured from Assiut University Mycological Centre (AUMC), Faculty of
Science, Assiut University, 71516 Assiut, Egypt. Potato dextrose agar medium was
used for fungi culturing. Culturing technique was prepared according to American
Type Culture Collection (1980).

**Preparation of samples for biochemical analysis:**

For biochemical analysis, insect bodies were homogenized using treatment
buffer (1 g insect body / 1 ml) in a chilled glass Teflon tissue grinder for 3 min
according to a modified (Laemmli, 1970) method. Homogenates were centrifuged at
14000 r.p.m for 15 min at 2°C in a refrigerated centrifuge. The supernatant was
separated for the biochemical determination. Five replicates were used for each
determination test. One replicate was used for the poly acrylamide gel
The Biochemical Effects of *B. bassiana* and *M. anisopliae* on 3rd instar larvae of *C. pipiens* L.

**Quantitative analysis of total protein:**

Total protein reagent was determined by the method of (Bradford, 1976), using Coommasie Brilliant Blue G-250 reagent (CBB). Sample solution of 50 µl was pipetted using a micropipette into a test tube and the volume was adjusted to 0.1 ml with phosphate buffer (pH 6.6). Five ml from protein reagent were added to the test tube and were mixed well. Absorbance at 595 nm was measured by using UV-1100 spectrophotometer (Chrom Tech, Inc.) after 2 minutes against blank prepared from 0.1 ml of phosphate buffer (pH 6.6) with 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance in the standard curve which used to determine the unknown protein concentration.

**Qualitative analysis of total protein:**

One-dimensional gel electrophoresis was performed in vertical polyacrylamide gels. Total larval proteins were profiled by SDS-polyacrylamide gel according to (Laemmli, 1970). SDS-molecular standard mixture of proteins; 245 kDa: 5 kDa (Sigma-Aldrich, Germany) was used as a marker with a 4% stacking gel and a 10% separating gel, at 100 volts for (5 hrs.) at room temperature. After electrophoresis, protein bands were stained for 2 hrs. in Coomassie brilliant blue (CBB.R-250), then destained for 24 hrs. for visualization of bands.

**Data analysis:**

The data were recorded, tabulated and subjected to statistical analysis using a software SPSS test program version 20 for Windows. The significance of the main effects was obtained by One-way analysis of variance (ANOVA) and followed by post-hoc analysis using LSD-test. The significance level of different treatments was evaluated at P < 0.05).

**RESULTS**

**Quantitative analysis of total protein of untreated and treated 3rd instar larvae of *C. pipiens*:**

The effect of sub-lethal concentrations (LC₅₀) of the two entomofungi on total protein content of the 3rd instar larvae of *C. pipiens* were recorded in (Table 1) and graphically illustrated in (Fig.1). Total protein contents were 9.23 ± 0.15 mg/ml and 3.6 ± 0.31 mg/ml in treated samples of *C. pipiens* larvae with *B. bassiana* and *M. anisopliae*, respectively as compared with 12.4 ± 0.23 mg/ml in the control samples. The total protein contents were significantly decreased after treatment with LC₅₀ of both fungi. The total protein contents were decreased by 26% and 71% in treated larvae with *B. bassiana* and *M. anisopliae*, respectively.

<table>
<thead>
<tr>
<th>Type of fungi treatment</th>
<th>Total protein in mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.4 ± 0.23 a</td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>9.23 ± 0.15 b</td>
</tr>
<tr>
<td><em>Metarhizium anisopliae</em></td>
<td>3.6 ± 0.31 c</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± SE
*Means with different letters within column are significantly different (P<0.05) ANOVA, LSD test.
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Fig 1: Changes in the concentration of total protein of normal and treated Culex pipiens larvae with the LC_{50} of two fungi (Beauveria bassiana and Metarhizium anisopliae). The asterisks show significant differences at (P <0.05). Confidence bars are revealed for ± SE.

Qualitative analysis of total protein of untreated and treated 3rd instar larvae of Cx. pipiens using polyacrylamide gel electrophoresis:

Changes in the protein fractionation profile of untreated and treated 3rd instar larvae of Cx. pipiens were analyzed after 48 hrs of treatment and recorded using (SDS-PAGE). The fraction electrophoretic protein patterns were shown in (Table 2), and presented in (Fig 2 & 3).

The SDS protein patterns of larval proteins showed different numbers of protein bands according to their molecular weights (Table 2). They were separated into 24 protein bands with molecular weight ranged from 263.07 to 4.88 kDa. The SDS protein electrophoresis revealed differences between the untreated and treated samples. The number of bands of treated samples with Beauveria bassiana and Metarhizium anisopliae were 14 and 9 bands, respectively compared with 21 bands in the control samples. Bands appeared in the control samples were with molecular weights ranged from 176.79 to 4.88 kDa. The total numbers of bands in samples treated with B. bassiana were with molecular weights ranged from 263.07 to 7.24 kDa.

The total numbers of bands in samples treated with M. anisopliae were with molecular weights ranged from 75 to 6.45 kDa.

There were 6 common bands between control and treated samples appeared in r7, r9, r13, r15, r21, and r22 with molecular weight approximately 67, 58, 23, 21, 11, and 7 kDa, respectively. There were 6 characteristic bands for the control sample with molecular weight 176.79, 104.18, 50.81, 31.93, 20.79, and 4.88 kDa and percentage amount 3.79, 3.45, 4, 4.41, 3.04, and 4.01%, respectively.

There was one characteristic band for the treated sample with B. bassiana with molecular weight 263.07 kDa and percentage amount 4.83%.

There was one characteristic band for the treated sample with M. anisopliae with molecular weight 6.45 kDa and percentage amount 4.78%.

Treatment with LC_{50} of B. bassiana caused the disappearance of 7 bands while treatment with M. anisopliae caused the disappearance of 12 bands.

The obtained results indicated that the application of entomofungi as larvicidal agents against mosquito larvae caused significant changes in the total protein profile of Cx. pipiens larvae.
The Biochemical Effects of *B. bassiana* and *M. anisopliae* on 3rd instar larvae of *C. pipiens* L.39

Fig 2: Electrophoretic protein pattern of SDS-PAGE of control and treated 3rd instar larvae of *Cx. pipiens* after 48 hrs. M: Marker, Lane 1: Control samples, Lane 2: Samples treated with LC_{50} *Beauveria bassiana*, Lane 3: Samples treated with LC_{50} *Metarhizium anisopliae*.

Fig 3: Denistometric scanning of SDS-electrophoresis protein patterns of control (Lane 1) and treated 3rd instar larvae of *Cx. pipiens* with LC_{50} *Beauveria bassiana* (Lane 2) and with LC_{50} *Metarhizium anisopliae* (Lane 3).
Table 2: Molecular weights of SDS-electrophoretic protein patterns for both untreated and treated samples of 3rd instar larvae of *Cx. pipiens* by *Beauveria bassiana* and *Metarhizium anisopliae* after 48 hrs.

<table>
<thead>
<tr>
<th>Rows</th>
<th>Marker (mol.w.)</th>
<th>Control (mol.w.)</th>
<th>Samples treated with <em>Beauveria bassiana</em> (mol.w.)</th>
<th>Samples treated with <em>Metarrhizium ansiopaliae</em> (mol.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r1</td>
<td>245</td>
<td></td>
<td>263.07</td>
<td></td>
</tr>
<tr>
<td>r2</td>
<td>180</td>
<td></td>
<td>176.79</td>
<td></td>
</tr>
<tr>
<td>r3</td>
<td>135</td>
<td></td>
<td>145.07</td>
<td>139.94</td>
</tr>
<tr>
<td>r4</td>
<td>100</td>
<td></td>
<td>104.18</td>
<td></td>
</tr>
<tr>
<td>r5</td>
<td></td>
<td></td>
<td>88.704</td>
<td>86.603</td>
</tr>
<tr>
<td>r6</td>
<td>75</td>
<td></td>
<td>75.904</td>
<td>75</td>
</tr>
<tr>
<td>r7</td>
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<td>67.624</td>
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</tr>
<tr>
<td>r8</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r9</td>
<td></td>
<td></td>
<td>60.272</td>
<td>58.028</td>
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<tr>
<td>r10</td>
<td>48</td>
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<td>r11</td>
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<td>r20</td>
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<td>12.621</td>
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<td>11</td>
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<td>10.026</td>
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<td>r24</td>
<td>5</td>
<td></td>
<td>4.8854</td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

Conventional insecticides play an important role in the overall Culex pipiens suppression program. However, development of Cx. pipiens resistance to various insecticides used indicated that great efforts should be made to find effective alternative methods of control.

Resistance to chemical insecticides is widely spread among large number of insect species (Georghiou and Mellon, 1983). In response to severe use of organophosphorus insecticides against Cx. pipiens mosquitoes, the resistance has increased basically upsetting control efforts (Bonning and Hemingway, 1991; Abou-El-Mahasen 2007).

Although fungal pathogens have much in common with viruses, bacteria, and other insect pathogenic microbes, they are distinctive in many ways (Ferron, 1978). Feasibly the most significant difference comes from the mode of infection; whereas most entomopathogens infect their hosts through the gut after feeding. Fungi typically penetrate the insect cuticle and considered major pathogens known to infect insects with sucking mouthparts, orders Hemiptera and Homoptera (Roberts and Humber, 1981).

In the present study total protein was quantitatively estimated in untreated 3rd instar larvae of Cx. pipiens and treated larvae with the sublethal concentration (LC50) of B. bassiana and M. anisopliae. Also qualitative analyses of treated 3rd instar larvae were studied. Total protein was conducted in a trial to detect the mode of action of the two entomopathogenic fungi.

Proteins are essential constituents of the general animal cells and also in the maintenance of different activities. These changes may be due to certain defects in enzymes that are responsible for protein and lipid synthesis. On the other hand, it is obvious that the tested biological agents decreased the protein contents in Culex pipiens larvae. Because proteins are essential to chitin synthesis, the depletion of this metabolic macromolecule indicates that chitin production must be inhibited. It is well known that the proteins are the major and in essential for the insect life (for energy production, for the adult fecundity, and for fertility) which were affected clearly by this biomatter.

As indicated from the obtained results, the protein contents of the treated larvae with LC50 of B. bassiana and M. anisopliae were significantly decreased in terms of the optical density measurements compared to the control samples. The obtained result agreed with Broome et al. (1976) who found that 0.36X10^8 spore/ml decreased the total protein content in adults of boll weevil, Anthonomus grandis by 41%. In agreement with our study, Sree and Joshi (2015) revealed that, inoculation of fungal pathogen Beauveria bassiana resulted significant reduction in protein content of haemolymph in treated silkworm larvae compared to control group. Also Elbanna et al., (2012) confirmed that total insect protein contents were radically declined with treatments of M. anisopliae fungi on locusts. Sahayaraj and Borgio (2010) found that the insecticidal activity of B. bassiana and M. anisopliae also reduced total body protein content in Dysdercus cingulatus.

The reduction in protein level resulted from damage of protein molecule and alteration of certain amino acid side chains due to damage of protein, which leads to alteration in its properties to the point where it can no longer serve its usual purpose (Spikes and Macknight, 1970). Broome et al., (1976) and Callaham et al., (1977) suggested that this decrease might be due to accumulative energy stress on the organism, and it may be having a great effect on adult mortality.
The changes in the protein profile of the treated *Cx. pipiens* larvae with LC50 of the 2 entomofungi were compared by electrophoretic runs of proteins extracted from treated and untreated larva. The electrophoretic analysis of SDS-PAGE protein pattern of the tissue proteins of control and treated samples of *Cx. pipiens*, with the sub-lethal dose of the two entomofungi showed changes in protein pattern. The total numbers of bands of control samples were 21 with molecular weights ranged from 176.79 to 4.88 kDa, while in treated samples with *B. bassiana* and *M. anisopliae* there were 14 and 9 bands, respectively. These data is in agreement with our obtained results of the total protein analyses. According to this study, SDS protein pattern suggested that, there were 6 common bands with molecular weight ranged between 67 and 7 kDa; these might be characteristics for *Cx. pipiens* samples as they were presented in both treated and control samples. Our results are in agreement with El-Sonbaty *et al.* (2016) who conveyed that analysis of haemolymph protein profile of *Spodoptera littoralis* larvae using gel electrophoresis showed fourteen differentially expressed proteins ranging from 9.6 - 116.2 KDa post infection with *M. anisopliae*. The infection distinctly affected protein profiles and can be manipulated in the proteins of molecular weight in the range of 56.9-82.6 KDa of prophenoloxidases and phenoloxidases. They concluded that *M. anisopliae* infection greatly affected cellular immune system and protein expression. So, it could result into death of insect due to disturbance in the immune system and proteins. Gabarty *et al.* (2013) reported that SDS protein analysis of the *Spodoptera littoralis* larvae revealed that the immune enzymes activity and protein concentration were significantly decreased at second, third, and fourth day of treatment with *Beauveria bassiana* and *Metarhizium anisopliae*.

It can be concluded from the obtained results that the application of entomofungi as larvicidal agents against mosquito larvae caused significant changes in the total protein profile of *Cx. pipiens* larvae suggesting that toxins secreted by these pathogens caused damage to the larval proteins which finally leads to larval death. This study recommends the use of *B. bassiana* and *M. anisopliae* as biological control agents to the control of *Cx. pipiens* larvae in order to suppress this medical vector for public health.

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The Biochemical Effects of *B. bassiana* and *M. anisopliae* on 3rd instar larvae of *C. pipiens*


ARABIC SUMMARY

التأثيرات البيوكيميائية لبيوفاريا بسيانا و متارزيم انزوبليو علي الطور البرقي الثالث لحشرة كيولكس بيبير (دوات الجناحين: كيوليسدي)

محمد علي محمود عيده* داليا عبد البديع سالم، فاطمة عبد ابراهيم سالم، نادية عبد الداليا ديوان
قسم علم الحشرات - كلية العلوم - جامعة عين شمس - القاهرة - مصر

تم اختبار التأثيرات البيوكيميائية لفطرين متخصصين في اصابة الحشرات (بيوفاريا بسيانا و متارزيم انزوبليو) في هذه الدراسة المقدمة في الطور البرقي الثالث لبعوض كيولكس بيبير في المختبر. أظهرت النتائج انخفاض معنوي في المحتوى البروتيني الكلي للطور البرقي للبعوض المعالج بالفطرين بمقارنة بالطور البرقي للعينات المرجعية.

أظهرت الدراسات البيوكيميائية باستخدام التبريد الكهربائي للبروتينات (SDS-PAGE) ان هناك انخفاض في عدد الحزم البروتينية للبروتينات المعالج بالفطرين.

وافترحت هذه النتائج أن السوؤل التي يفرزها الجراثيم تسببت في أضرار في بروتينات البروتينات، مما يؤدي إلى موتها.

والاستنتاج أن مسببات الأمراض الفطرية هامة في مجال الكبايدة البيولوجية الطبيعية ضد العديد من الحشرات وغيرهما وتشير إلى أن عدود كبير من الأفاف. وتمت هذه الدراسة استخدام فطرين بسيانا و متارزيم انزوبليو لعملية مكافحة بيولوجية لبعوض كيولكس بيبير بشكل أمن من أجل قمع هذا الحشرة الطبية ومن أجل الصحة العامة.