Original Research Article

Larvicidal activity of entomopathogenic fungi *Metarhizium anisopliae* against mosquito larvae in Algeria

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**ABSTRACT**

The entomopathogenic fungi *Metarhizium anisopliae* has demonstrated its efficiency against mosquito species in the laboratory. The virulence of *Metarhizium anisopliae* was tested against 4th instar larvae of *Culex pipiens* using five concentrations $10^9$, $10^8$, $10^7$, $10^6$ and $10^5$ conidia/ml. The larval mortalities were observed for a period of 10 days. The mortality values were then subjected by the probit analysis. LC$_{50}$ and LC$_{90}$ values were calculated. Results showed that the mortality of mosquito larvae treated with the different fungal concentrations varied from 4 to 96%. No mortality was observed in the control. Larvae mortality rate increased with increasing conidia concentration. The bioassays showed LC$_{50}$ values after 24, 48, 72 and 96 hours of exposure were $3.9 \times 10^8$, $7.5 \times 10^7$, $7.7 \times 10^6$ and $5.1 \times 10^5$ conidia/ml respectively while LC$_{90}$ values calculated were $1.9 \times 10^{11}$, $2.8 \times 10^{10}$, $1.8 \times 10^9$ and $1.5 \times 10^8$ conidia/ml respectively. The results indicate that *Metarhizium anisopliae* has the potential to be a biocontrol agent for *Culex pipiens* and is suitable candidate for further research and development.

**Keywords**

Biocontrol; *Metarhizium anisopliae*; Entomopathogenic fungi; *Culex pipiens*; Larvicidal pathogenicity.

**Introduction**

Mosquitoes are a serious threat to public health transmitting several dangerous diseases in over 2 billion people in the tropics (Odalo et al., 2005). Mosquito control and personal protection from mosquito bites are currently the most important measures employed to control these diseases. Many approaches have been developed and that aim to diminish mosquito menace. The use of larvicides and repellents can be an economical and practical way to prevent the transmission of these diseases to humans. The common approach for the control of mosquito vectors and reducing the transmission of human pathogens is based on the chemical insecticides (Paul et al., 2006). The use of chemical insecticides is still the most important element in mosquito control programmes (Alves et al., 2002). The drawbacks associated with the chemical insecticides, e.g., resistance in vector population, environmental pollution and costs have led to the search for alternative
control agents. In recent years, efforts have been made on the search for natural products derived from plants and microorganisms as an alternative to conventional chemical insecticides for insect-control (Quesada-Moraga et al., 2006).

Many biological control agents have been evaluated to determine if they are pathogenic to larval stages of mosquitoes, of which the most successful include bacteria such as *Bacillus thuringiensis israelensis* and *Bacillus sphericus* (Fillinger et al., 2003).

However, entomopathogenic fungi are considered excellent candidates for biopesticides due to their safety, relatively limited host range, ease of production and suitability of large scale production (Ferron 1981).

Entomopathogenic Ascomycetes (notably *M. anisopliae* and *B. bassiana*) are among the most commonly encountered insect pathogens (Goettel and Inglis 1997), and are in use to manage various arthropod pest species (Bartlett and Jeronski 1988; Federici 1995; Zimmermann 1993; Khetan 2001).

*Metarhizium anisopliae*, discovered 125 years ago by Mechnichoff has a rather wide host range. It is widely used as a biocontrol agent on various types of pests, including mosquito larvae.

The use of entomopathogenic fungi against a range of mosquito larvae has been the subject of various studies (Clark et al., 1988; Alves et al., 2002). Studies demonstrated that entomopathogenic fungi can be effective at killing mosquito larvae under laboratory conditions but were highly variable when tested in the field, suggesting the need for a greater understanding of epizootiology (Goettel 1987a). In the case of the Dengue vector *Ae. aegypti*, the biology of this species would appear to favor the use of entomopathogenic fungi (Scholte et al. 2004).

The objective of this study was to evaluate pathogenicity of the fungus *Metarhizium anisopliae* against fourth instars larvae of *Culex pipiens* under laboratory conditions.

**Materials and Methods**

**Isolation and identification of entomopathogenic fungi**

*Metarhizium anisopliae* was isolated from the agricultural soil samples of Ain el Bey, Constantine, Algeria. Serial dilutions were made up to five dilutions. From each dilution was plated on DOA (dodine oatmeal agar) selective medium for screening entomopathogenic fungi (containing 200 µg/ml dodine (Beilharz et al., 1982) and 50 µg/ml streptomycine (Chase et al., 1986, Liu et al., 2007; Du et al., 2008). The plates were incubated for 14 days at 28°C. After 14 days resulting colonies were purified on SDAY (Sabouraud dextrose agar) and identified using standard mycological keys.

**Fungus culture**

Isolates of *Metarhizium anisopliae* were cultures on PDA (potato dextrose agar) medium and incubated at 28°C for 14 days after inoculation. The conidia were harvested by scraping the surface of 14 days old culture and were suspended in solution of 0.01% Tween80 in distilled water. The mixture was stirred with a magnetic stirrer for 10 min. The conidial concentration of the final suspension was
determined by direct count using a haemocytometer (Hazrat et al., 2012). Dilutions were made using 0.01% Tween80 to obtain conidia concentrations of $10^9$, $10^8$, $10^7$, $10^6$ and $10^5$ conidia/ml.

Mosquito's larvae rearing

*Culex pipiens* larvae were maintained in the laboratory at a temperature of 27 + 2°C, relative humidity of 70 + 5% and a photoperiod of 14:10h. Different instars of mosquitos were maintained inseparate enamel container at a density of 200 larave per container. Larvae were provided a mixture of yeast powder and biscuit as food every 24 hours. Larvae were reared in distilled water at pH 7.0. To counteract evaporation, water was added daily.

Larvicidal bioassays

Conidia of *Metarhizium anisopliae* were tested against mosquitoe larvae by adding fungal suspension to plastic cups containing 50 ml of distilled water with 25 larvae of the 4th instar. Each cup was inoculated with 1ml of fungal suspensions ($10^9$, $10^8$, $10^7$, $10^6$ and $10^5$ conidia/ml). Control treatments were carried out by addition of 10 ml of distilled water. Each assay was conducted three times. Larvae were fed with cat croquette and they were observed daily, larvac mortality was evaluated on a daily basis of 10 days.

Statistical analysis

The data on the efficacy were subjected to probit analysis (Finney 1971). The relationships between probit and log concentrations were established as probit equations and probit regression lines were drow.

Result and Discussion

The efficacy of *Metarhizium anisopliae* was assessed against 4th instars larvae of *Culex pipiens* at various conidia concentrations $10^5$ to $10^9$ in different time period. When the above five concentrations of fungus were applied on 4th instars larvae, it was observed that mortality increased as the time period was increased. The percentage mortality of mosquito larvae varied from 4 to 96% as shown in table1. Revealed maximum of mortality at highest applied dose of $10^9$ conidia/ml. Whereas it was 40% with lowest dose level of $10^5$ conidia/ml and again 48.76 and 88 % at $10^6$, $10^7$ and $10^8$ conidia/ml respectively. But the mortality increased from 52% to 96% at highest dose of $10^9$ conidia/ml from 24 hours to 96 hours respectively (Fig 1). This indicate that, for some concentrations, fungus isolate was taking time to kill 96% tested larvae.

The efficacy of conidia of *Metarhizium anisopliae* at the 4th instars larvae was expressed in terms of LC50 and LC90. Regarding the efficacy of *Metarhizium anisopliae* against mosquito larvae, it was found that the LC50 value was observed as $3.9.10^8$ conidia /ml after 24h, while the LC90 value observed was $1.9.10^{11}$ conidia/ml. Moreover, after 48h, the calculated LC50 value was $7.5.10^7$ conidia/ml. The calculated LC90 value was $2.8.10^{10}$ conidia /ml, after 72h, the LC50 value was observed as $7.7.10^6$ conidia /ml and the LC90 value observed was $1.8.10^9$conidia /ml. After 96h, the LC50 value was recorded as $5.1.10^5$ conidia/ml; for the LC90 value these was recorded as $1.5.10^8$conidia/ml (Table 2; Figs 2,3, 4 and 5).
Table 1 Percentage mortality of *Culex pipiens* fourth instar larvae exposed different concentrations of *Metarhizium anisopliae* isolate

<table>
<thead>
<tr>
<th>Concentration (Conidia/ml)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 Hours</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>$10^5$</td>
<td>4</td>
</tr>
<tr>
<td>$10^6$</td>
<td>8</td>
</tr>
<tr>
<td>$10^7$</td>
<td>32</td>
</tr>
<tr>
<td>$10^8$</td>
<td>40</td>
</tr>
<tr>
<td>$10^9$</td>
<td>52</td>
</tr>
</tbody>
</table>

Figure 1 Larval mortality (%) at different concentration of *Metarhizium anisopliae*

Table 2 The efficacy of *Metarhizium anisopliae* against mosquito larvae after 24, 48, 72 and 96 hours

<table>
<thead>
<tr>
<th>Time of exposure</th>
<th>Probit equation</th>
<th>$LC_{50}$ conidia/ml</th>
<th>$LC_{90}$ conidia/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>$0.477x + 0.897$</td>
<td>$3.9.10^8$</td>
<td>$1.9.10^{11}$</td>
</tr>
<tr>
<td>48 hours</td>
<td>$0.499x + 1.063$</td>
<td>$7.5.10^7$</td>
<td>$2.8.10^{10}$</td>
</tr>
<tr>
<td>72 hours</td>
<td>$0.539x + 1.283$</td>
<td>$7.7.10^6$</td>
<td>$1.8.10^9$</td>
</tr>
<tr>
<td>96 hours</td>
<td>$0.521x + 2.023$</td>
<td>$5.1.10^5$</td>
<td>$1.5.10^8$</td>
</tr>
</tbody>
</table>
**Figure 2** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4th instars of mosquito larvae after 24 hours.

![Probit regression line for 24h](image1)

**Figure 3** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4th instars of mosquito larvae after 48 hours.

![Probit regression line for 48h](image2)

**Figure 4** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4th instars of mosquito larvae after 72 hours.

![Probit regression line for 72h](image3)
**Figure 5** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4th instars of mosquito larvae after 96 hours

*Metarhizium* is one of the most common entomopathogenic fungi with a worldwide distribution. The species is soil-borne and infects predominantly soil-dwelling insects.

Mosquitoes are not listed as natural hosts for *Metarhizium anisopliae* (Scholte et al., 2004) but some strains have shown to be virulent against mosquito larvae (Roberts 1967, 1970, 1974; Ramoska 1982; Daoust and Roberts 1983a,b; Agudelo-Silva and Wassink 1984; Sandhu et al., 1993; Alves et al., 2002; Scholte et al., 2005; Amora et al., 2010).

Many studies have shown the potential of *Metarhizium anisopliae* as a mosquito control agent. Researchers observed effects of this fungus on larvae of *Anopheles stephensi*, *Anopheles quadrimaculatus*, *Aedes aegypti*, *Ochlerotatus atropalpus*, *Ochlerotatus taeniorhynchus*, *Culex pipiens*, *Culex restuans*, and *Culex salinarius* (Roberts 1970). Furthermore, it showed that *Metarhizium anisopliae* fungus can successfully infect and kill larvae of *Ae. aegypti* and *C. quinquefasciatus* with 100% of mortality (Mc Cray et al., 1973).

The present experiment was carried out for the evaluation of isolated fungus *M. anisopliae* against *C. pipiens* larvae as immature vectors which is the most perfect stage for the bio-control agents. In our study of the virulence of *M anisopliae* against larvae *C. pipiens*, a significant mortality was observed. Indeed, the percentage mortality of mosquito larvae reached 96%. These results are similar to these by Daoust and Roberts (1982) who reported a mortality greater than 90% on 30% of its isolates. Also, the percentage larval mortality was enhanced significantly when increasing concentration and time. Our results also proved by Scholte et al., (2003) and Blanford et al., (2005), that fungus isolates take time to kill different mosquito species but that depending upon the dose and fungus strain.

A number of entomopathogenic fungi have been so far used effectively to control mosquito vector for the last decades. They are unique because fungi have the ability
to directly infect the host by penetration into the cuticle and do not need to ingest by the insect to cause disease. *Metarhizium anisopliae* has so far not been tested in Algeria and this is the primary report on it as mosquito larvicide. Further studies of larvicidal activity of this fungus suggest that it is a good biocontrol agent. However, this isolate can be used in mosquito control programs, in Algeria, alone or perhaps in combination with other control agents such as *Bacillus thuringiensis* or *Bacillus sphaericus*.

References


Ferron, P., 1981. Pest control by the fungi


