

## Original Research Article

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

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## ABSTRACT

### Keywords

Biocontrol;  
*Metarhizium anisopliae*;  
Entomo -  
pathogenic  
fungi;  
*Culex pipiens*;  
Larvicidal  
pathogenicity.

The entomopathogenic fungi *Metarhizium anisopliae* has demonstrated its efficiency against mosquito species in the laboratory. The virulence of *Metarhizium anisopliae* was tested against 4<sup>th</sup> 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  $3.9 \cdot 10^8$ ,  $7.5 \cdot 10^7$ ,  $7.7 \cdot 10^6$  and  $5.1 \cdot 10^5$  conidia/ml respectively while  $LC_{90}$  values calculated were  $1.9 \cdot 10^{11}$ ,  $2.8 \cdot 10^{10}$ ,  $1.8 \cdot 10^9$  and  $1.5 \cdot 10^8$  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.

## 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 use of 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 streptomycin (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 cultured 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  $27 \pm 2^\circ\text{C}$ , relative humidity of  $70 \pm 5\%$  and a photoperiod of 14:10h. Different instars of mosquitos were maintained in separate enamel container at a density of 200 larvae 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 mosquito larvae by adding fungal suspension to plastic cups containing 50 ml of distilled water with 25 larvae of the 4<sup>th</sup> 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, larvae 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 drawn.

## Result and Discussion

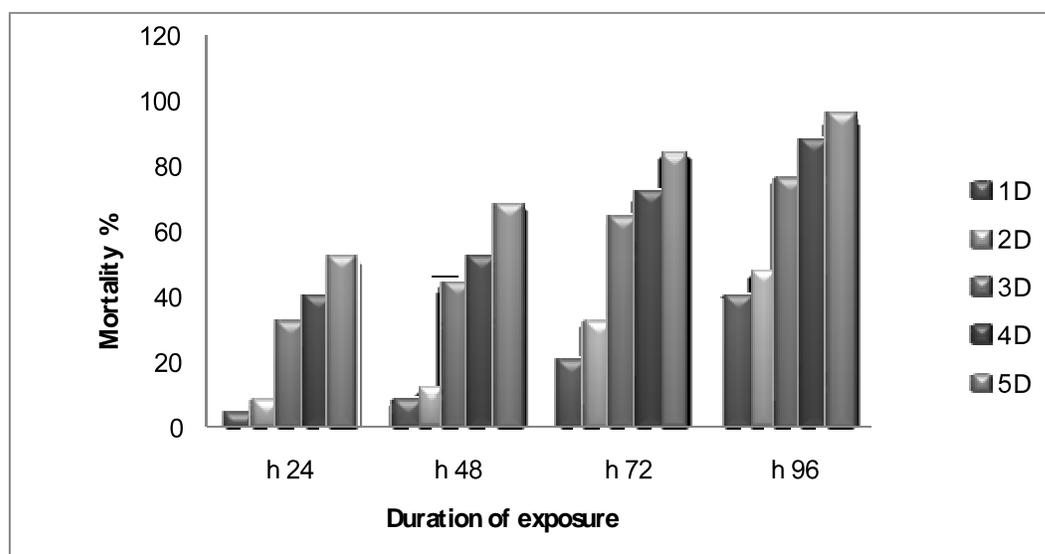
The efficacy of *Metarhizium anisopliae* was assessed against 4<sup>th</sup> instars larvae of *Culex pipiens* at various conidia concentrations  $10^5$  to  $10^9$  in different time period. When the above five concentrations of fungus were applied on 4<sup>th</sup> instars larvae, it was observed that mortality increased as the time period was increased. The percentage mortality of mosquito larvae varied from 4 to 96% as shown in table 1. 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 indicates that, for some concentrations, fungus isolate was taking time to kill 96% tested larvae.

The efficacy of conidia of *Metarhizium anisopliae* at the 4<sup>th</sup> 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  $3.9 \cdot 10^8$  conidia/ml after 24h, while the LC90 value observed was  $1.9 \cdot 10^{11}$  conidia/ml. Moreover, after 48h, the calculated LC50 value was  $7.5 \cdot 10^7$  conidia/ml. The calculated LC90 value was  $2.8 \cdot 10^{10}$  conidia/ml, after 72h, the LC50 value was observed as  $7.7 \cdot 10^6$  conidia/ml and the LC90 value observed was  $1.8 \cdot 10^9$  conidia/ml. After 96h, the LC50 value was recorded as  $5.1 \cdot 10^5$  conidia/ml; for the LC90 value these was recorded as  $1.5 \cdot 10^8$  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

Concentration (Conidia/ml )	Time			
	24 Hours	48 Hours	72 Hours	96 Hours
Control	0	0	0	0
10 <sup>5</sup>	4	8	20	40
10 <sup>6</sup>	8	12	32	48
10 <sup>7</sup>	32	44	64	76
10 <sup>8</sup>	40	52	72	88
10 <sup>9</sup>	52	68	84	96

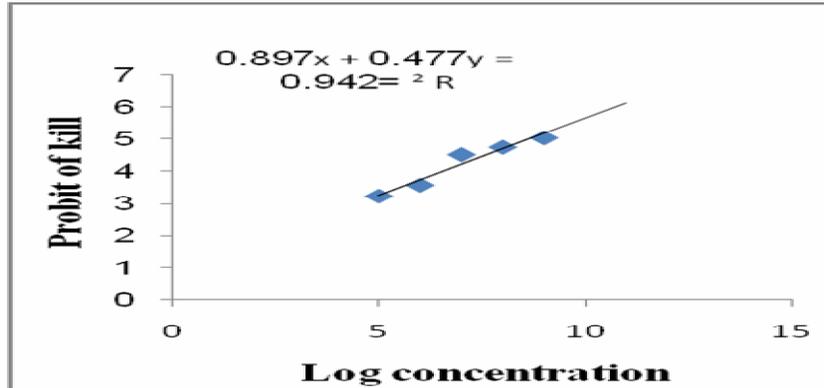
**Figure.1** Larval mortality (%) at different concentration of *Metarhizium anisopliae*



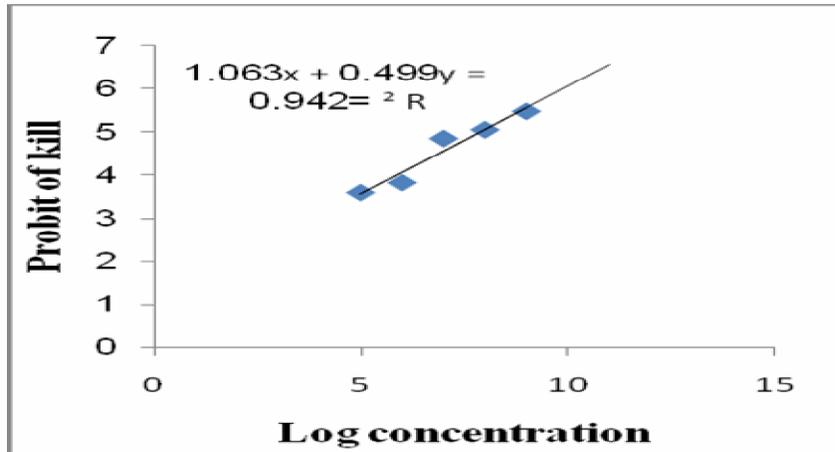
**Table.2** The efficacy of *Metarhizium anisopliae* against mosquito larvae after 24, 48, 72 and 96 hours

Time of exposure	Probit equation	LC <sub>50</sub> conidia /ml	LC <sub>90</sub> conidia/ml
24 hours	0.477x + 0.897	3.9.10 <sup>8</sup>	1.9.10 <sup>11</sup>
48 hours	0.499x + 1.063	7.5.10 <sup>7</sup>	2.8.10 <sup>10</sup>
72 hours	0.539x + 1.283	7.7.10 <sup>6</sup>	1.8.10 <sup>9</sup>
96 hours	0.521x + 2.023	5.1.10 <sup>5</sup>	1.5.10 <sup>8</sup>

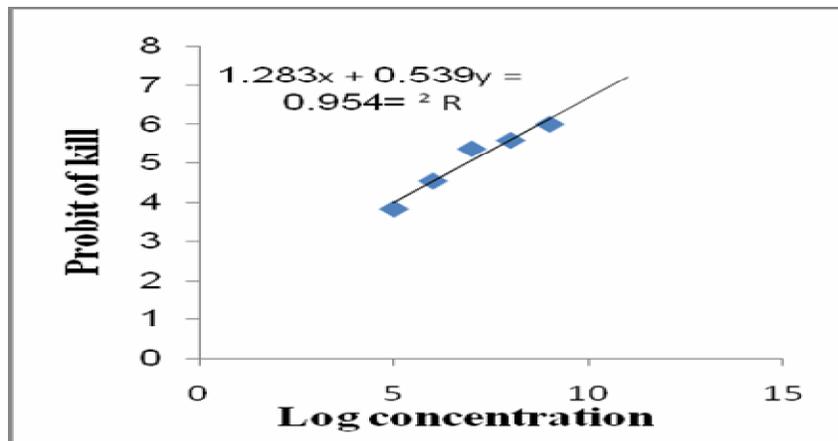
**Figure.2** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4<sup>th</sup> instars of mosquito larvae after 24 hours



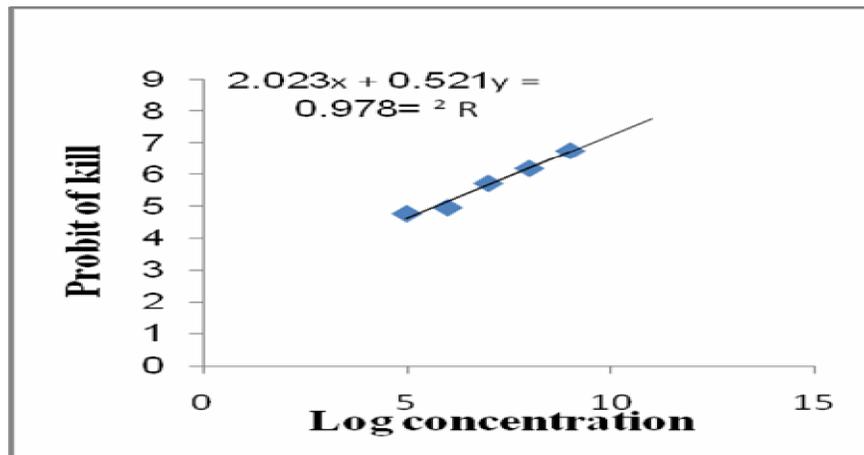
**Figure.3** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4<sup>th</sup> instars of mosquito larvae after 48 hours.



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



**Figure.5** Probit regression line depicting the relation between probit of kill and log dose concentration of *Metarhizium anisopliae* against 4<sup>th</sup> 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.* Unlike other mosquito control agents, they

*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. anisoplia* 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 those 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. 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

- Agudelo-Silva, F., and Wassink, H. 1984. Infectivity of a Venezuelan strain of *Metarhizium anisopliae* to *Aedes aegypti* larvae. J. Invertebr. Pathol. 435-436.
- Alves, S.B. Alves, L.F.A. Lopes, R.B. Pereira, R.M. Vieira and S.A. 2002. Potential of Some *Metarhizium anisopliae* Isolates for Control of *Culex quinquefasciatus* (Dipt. Culicidae). J. Entomo. 126: 504-509.
- Amóra, S.S.A., Bevilaqua, C.M.L. Feijó, F.M.C. Pereira, R.H.M.A. Alves, N.D. Freire, F.A.M. Kamimura, M.T.K. De Oliveira, D.M. Lima, E.A.L.A and Rocha. M.F.G. 2010. The effects of the fungus *Metarhizium anisopliae* var. *acidum* on different stages of *Lutzomyia longipalpis* (Diptera: Psychodidae). Acta Tropica 113: (3) 214-220
- Bartlett, M.C., and Jeronskim, S.T. 1988. Mass production of entomogenous fungi for biological control of insects In: Burge, M.N. (ed.), *Fungi in Biological Control Systems*. 61-85. Manchester University Press, Manchester, United Kingdom.
- Beilharz, V.C., Parbery, D.G and Swart. H.J. 1982. Dodine: A selective agent for certain fungi. Transaction of British. Myco Societ. 79:(3) 507-511.
- Blanford, S., 2005. Fungal pathogen reduces potential for malaria transmission. Science. 308: 1638-1641.
- Chase, A.R., Osborne, L.S and Ferguson, V.M. 1986. Selective isolation of the entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium. Florida. Entomologist. 69:(2) 285-292
- Clark, T.B. Kellen, W. Fukuda, T. Lindegren, J.E. 1968. Field and laboratory studies on the pathogenicity of the fungus *Beauveria bassiana* to three genera of mosquitoes. J. Invertebr. Pathol. 11: 1-7.
- Daoust, R.A., Ward, M.G. and Roberts, D.W. 1982. Effect of formulation on the virulence of *Metarhizium anisopliae* conidia against mosquito larvae. J. Invertebr. Pathol. 40:228-236.
- Daoust, R.A., and Roberts, D.W. 1983a. Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of temperature and relative humidity on conidial viability and virulence against mosquitoes. J. Invertebr. Pathol. 41: 143-150.
- Daoust, R.A., and Roberts, D.W. 1983b. Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of growth substrate on conidial survival and virulence against mosquitoes. J. Invertebr. Pathol. 41: 161-170.
- Du, K.S., Chai, L.Y. Xu, Y.L and Lang, J.F. 2008. Isolation of a *Metarhizium* Strain. J. Microbio. 28(6): 57-60.
- Federici, B.A., 1995. The future of microbial pesticides as vector control agents. J. Americ. Mosquito Control Association. 11: 260-268
- Ferron, P., 1981. Pest control by the fungi

- Beauveria* and *Metarhizium*. In: Burgess HD, editor. *Microbial Control of Pests and Plant Diseases 1970-1980*. 24: 465-482. London, Academic Press.
- Fillinger, U., Knols, B.G.J and Becker, N. 2003. Efficacy and efficiency of new *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* formulations against the malarial vector *Anopheles gambiae* in Western Kenya. *Tropic. Med. Internat. Health*. 8: 37- 48.
- Finney, D.J., 1971. Probit analysis, 3rd edn. Cambridge University Press, Cambridge, UK.
- Goettel. M.S., 1987a. Preliminary Field Trials with the Entomopathogenic Hyphomycete *Tolypocladium cylindrosporum* in Central Alberta. *J. American Mosquito Control Associatio*. 3: 239- 245.
- Goettel, M.S., and Inglis, G.D. 1997. Fungi: Hyphomycetes. In: Lacey, L.A. (ed.) *Manual of Techniques in Insect Pathol.* 213-249. Academic Press, San Diego, USA
- Hazrat, B., Soaib Ali, and H. Imtinan, A.K . 2012. Isolation and efficacy of entomopathogenic fungus *Metarhizium anisopliae* for the control of *Aedes albopictus* Skuse larvae: suspected dengue vector in Pakistan. *Asian Pacific J. Tropic. Biomed.* 298-300
- Kannan, S.K., Murugun, K. Kumar, AN. Ramasubramanian and N. Mathiyazhagan, P. 2008. Adulticide effect of fungal pathogen, *Metarhizium anisopliae* on malaria vector *Anopheles stephensi* (Diptera: Culicidae), *Afric J. Biotechno.* 7: 838-841.
- Khetan, S.K ., 2001. Mycoinsecticides. In: Khetan, S.K. (ed.) *Microbial pest control*. 211-222. Marcel Dekker, Inc. Basel, Switzerland.
- Liu, S.F., Z.H. Ye and Jiang, S.R. 2007. Isolation and Virulence Test of *Metarhizium*. *J. Anhui Agric. Sci.* 35(17): 5058-5059, 5077.
- McCray E.M, Jr. Womelderoff, D.J. Husbands, R.C and Eliason, D.A. 1973. Laboratory observation and field tests with *Legenidium* against California Mosquitoes. *Proceed. Califo. Mosquito Control Association* 41:123–128
- Miranpuri, G.S., and Khachatourians, G.G. 1991. Infection Sites of the Entomopathogenic Fungus *Beauveria bassiana* in the Larvae of the Mosquito *Aedes aegypti*. *Entomologia Experimentalis et Applicata*, 59 :19-27
- Odalo, J., Omolo , M. Malebo, H. Angira, J. Njeru, P. Ndiege, I. *et al.*, 2005. Repellency of essential oils of some plants from the Kenyan coast against *Anopheles gambiae*. *Acta.Tropica.* 95: 210-218 .
- Paul, A., Harrington, L.C. and Scott, J.G. 2006. Evaluation of novel insecticides for control of dengue vector *Aedes aegypti* (Diptera: culicidae). *J. Medi. Entomo.* 43: 55-60.
- Quesada-Moraga,E., Carrasco-Díaz,J.A and Santiago-Alvarez. C. 2006. Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae). *J. Appl. Entomo.* 130:442-452
- Ramoska, W.A., 1982 An examination of the long-term epizootic potential of various artificially introduced mosquito larval pathogens. *Mosquito News.* 42: 603-607.
- Roberts, D.W., 1967 Some effects of *Metarhizium anisopliae* and its toxins on mosquito larvae. In: Van der Laan, editor. *Insect Pathology and Microbial*

- Control. Amsterdam. 243-246. North-Holland Publishing Company.
- Roberts, D.W., 1970. *Coelomomyces*, *Entomophthora*, *Beauveria*, and *Metarrhizium* as parasites of mosquitoes. *Miscellaneous Publications of the Entomological Society of America* 7: 140-155.
- Roberts, D.W., 1974. Fungal infections of mosquitoes. In: Aubin A, Belloncik S, Bourassa JP, LaCoursière E, Péllissier M, editors. *Le contrôle des moustiques/Mosquito control*. 143-193. Presses de l'Université du Québec.
- Sandhu, S.S. ,Rajak, R.C. and Sharma, M. 1993. Bioactivity of *Beauveria bassiana* and *Metarhizium anisopliae* as pathogens of *Culex tritaeniorhynchus* and *Aedes aegypti*: effect of instar, dosages and time. *Indian J. Microbiol.* 33 : 191-194.
- Scholte, E.J., Takken, W. and Knols, B.G.J. 2003. Pathogenicity of six east African entomopathogenic fungi to adult *Anopheles gambiae* s.s. (Diptera: Culicidae) mosquitoes. *Proceedings of Experimental and Applied Entomology NEV*, Amsterdam. 14 : 25-29
- Scholte, E.J., Knols, B.G.J.K. Samson, R.A and Takken, W. 2004. Entomopathogenic fungi for mosquito control: a review. *J. Insect Sci.* 4:1.
- Scholte , E.J., Ng`habi, K. Kihonda, J. Takken, W. Paaijmans, K. Abdula, S.Killeen, G.F and Knols, B.G.J. 2005. An Entomopathogenic Fungus for Control of Adult African Malaria Mosquitoes', *Science*. 308: 1641-1642.
- Zimmermann ,G., 1993. The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. *Pesticide Sci.* 37: 375-379.