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Original article

Effect of the entomopathogenic fungus *Metarhizium anisopliae* against Mosquitos (*Aedes aegypti*) in vitro conditions

M. N. Yakubu [a, *], F. M. Adamu [a], A. A. Barde [b], M. Muhammad [a] & F. Tahir [c]

[a] Department of Applied Ecology, Abubakar Tafawa Balewa University, Bauchi, Nigeria.
[b] Department of Biological Sciences, Abubakar Tafawa Balewa University, Bauchi, Nigeria.
[c] Department of Microbiology, Abubakar Tafawa Balewa University, Bauchi, Nigeria.

Abstract

Entomopathogenic fungi are promising new biological tool for the control of pests and pose no risk to man and non-targeted organisms. Isolation and identification of native entomopathogenic fungi in a field is necessary for the successful control of pest in a particular region.

Experiment was conducted to evaluate the occurrence of entomopathogenic fungi from different soil and their efficacy in the control of mosquitos. Using rain beetle, the fungi were isolated by insect baiting method using PDA. Morphological identification of the isolated fungi showed *Metarhizium anisopliae*. The result of the study showed a variation in the occurrence of the *Metarhizium anisopliae* at the three locations. The pathogenicity of the isolated fungi was tested on *Aedes eagyptii* larvae in three different concentrations ($1.8.10^3$, $3.6.10^3$ and $4.5.10^3$ conidia/ml). The result showed a progressive increased in larval mortality with an increased number of days. The mortality varied from 4 to 100%. Rapid larval mortality was observed two days after spraying, mortality was highest and relatively uniform at higher concentration. No mortality was observed in the control treatments. The test showed LC_{50} value of $14.5.10^3$, $12.9.10^3$, $1.4.10^3$, $0.4.10^3$, $0.4.10^3$ and $0.1.10^3$ conidia/ml after exposure for 1, 2, 3, 4, 5, and 6 days respectively.

The outcome of this research showed variation in the diversity of entomopathogenic fungi in different soil location. *Metarhizium anisopliae* look promising in the control of the *Aedes eagyptii* mosquito larvae and should be tested in the field, or this could be the starting point for a genetic experiment.

Keywords: *Metarhizium anisopliae, Aedes eagyptii,* biocontrol

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*** Corresponding author:**

Mohammed Nuru Yakubu, Department of Applied Ecology, Abubakar Tafawa Balewa University, Bauchi, Nigeria.
Email: nooryaqub002@gmail.com

INTRODUCTION

Mosquito is a parasite of global importance causing a serious menace, spreading malaria, dengue and yellow fever which are collectively responsible for millions of deaths every year (WHO, 1996). Among all the diseases transmitted by mosquito dengue fever is considered to be the most important mosquito-borne viral disease in the world (Araujo *et al.,* 2015). *Aedes eagyptii* is the primary vector of dengue, yellow fever, and Zika flaviviruses (Harry *et al.,* 2018). World Health Organization reported that 40% of the world population is at the risk of contracting dengue and it can greatly affect global economy (Darbro *et al*., 2011). The global impact of dengue fever is high with an estimated to cases of 50 million per year (WHO, 2009) and re-estimation to 400 million cases (Bhatt *et al*., 2013) in over 100 countries (Darbro *et al*., 2011). Incidence of dengue fever is increasing, affecting an approximately 500,000 patients worldwide causing an estimated mortality of 2.5% of cases (WHO, 2011). However, Zika fever is another major human disease transmitted by *Aedes eagyptii*. Zika fever was declared a Public Health Emergency of International Concern by World Health Organization as a result of serious pandemic in America (Fauci and Morens, 2016).

Till date no available control for dengue fever, vector control is the dominant tool to reduce the incidence of this disease (Darbro *et al*., 2011)**.**

In recent years an unprecedented progress has been achieved in malaria prevention and control. However, this success is threatened by developed resistance to insecticides among mosquitos' parasites. Insecticide resistance could lead to increase in malaria incidence and mortality (WHO, 2017). Several studies reported *Aedes eagyptii* resistance (Hemingway and Ranson, 2000; Ponlawat, *et al.,* 2005; Garcı´a *et al.,* 2009) to chemical pesticides. The rapid acceleration of chemical pesticides in agricultural production has in many cases lead to increased production, but it has also had several adverse effects; deteriorating the environment in different ways such as contaminating water source and bottom sediments. Insect pest are becoming more resilient to many pesticides and the pesticides can impact non-target organism negatively such as animals and humans (Amuwitagama, 2004). The Swedish National Food Administration revealed that one-third of the samples from cereals, fruits and vegetables are contained with traceable amounts of at least two pesticides. Pesticides have many disadvantages such as harming non – target organism (Messmer & Dahl, 2009). The emergence of mosquito resistance to chemical parasite has renewed interest in alternative eco-friendly control measures. Among them biological control is one of the most effective alternatives. The use of fungi in biological control reduced the insect population and damage on crops. Entomopathogenic fungi as biological control agent have numerous advantages compared to insecticides. These advantages are eco-friendly for beneficial organisms, low cost, efficiency, reduction of residues accumulation in the environment and increased biodiversity in the ecosystem (Kaushal *et al.,* 2016)

The use of entomopathogenic fungi against a range of mosquito larvae has been the subject of various studies (Alves, *et al.,* 2002). Benserradj, *et al.,* (2014) showed that entomopathogenic fungi effectively killed mosquito larvae under laboratory conditions but were highly variable when tested in the field. The major limitation of the use of entomopathogenic fungi is the lack of persistent of the infective spore stage (Alves *et al.,* 2002; Scholte *et al*., 2004). These suggested the necessity for greater understanding of epizootiology (Goettel, *et al*., 2001). Mosquitoes are not mentioned among the natural hosts for *Metarhizium anisopliae* (Scholte *et al.,* 2004). However, some strains have shown to be virulence against mosquito larvae (Sandhu *et al.,* 2000; Alves *et al*., 2002; Scholte *et al*., 2005; Amora *et al.,* 2010; Benserradj *et al*., 2014).

Studies have demonstrated *Metarhizium anisopliae* and *Beauveria bassiana* are effective in the control of several agricultural insect pests (Arthurs & Thomas, 2000). These fungi can successfully infect and kill insects without being consumed. The conidia of the fungi attach to the insect's external tegument in a passive and non-specific way and subsequently germinate and penetrate the cuticle. When the conidia penetrate into the insect hemocoel, the mycelium spread throughout the host, forming hyphal bodies called blastospores (Scholte *et al*., 2004). The fungi produce toxins causing paralysis (Goettel and Inglis, 2001) and insects die within fourteen days of infection, depending on species, size and fungal isolate. Hyphae emerges from the dead insect and produce conidia on the exterior of the host under favorable condition. The conidia can be dispersed by wind or water (Marit and Bart, 2007). Using these ubiquitous fungi as a biological control agent within their natural environment will cause limited harm on the delicate ecological equilibria. Recent studies demonstrated that fungal infection in adult mosquitoes reduced longevity (Scholte *et al*., 2003; Scholte *et al*., 2005, Blandford, *et al.,* 2005). More recently Farenhorst *et al.,* (2009) demonstrates that fungal pathogens can infect insecticide resistance mosquitoes.

It is therefore not surprising that interest in alternative non-chemical strategies has increased over the last decades (Scholte *et al*., 2004).

Entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agriculture, pasture, desert and urban habitats (Lacey *et al.,* 2015). Their ability to regulate insect populations has been studied in tropical and temperate habitats (Sergio *et al.,* 2011)

Soil is considered an excellent environment shelter for entomopathogenic fungi since it is protected from Ultraviolet radiation and other adverse abiotic and biotic influences (Sergio *et al*., 2011).

A large amount of genetic diversity has been reported in entomopathogenic fungi (Bidochka, *et al.,* 2001), and the potential existence of strains adapted to various hosts, environmental conditions, conidial survival and competitive saprophytic ability can profoundly influence their virulence. Studies

have shown that strains can be distinguished by their different levels of proteases, chitinases and lipases (Eilenberg and Hokkanen, 2006).

Chemical pest control is still the common type of pest control today, even though its long-term effects led to a renewed interest in traditional and biological pest control. Entomopathogenic fungi are natural enemies of insects and arachnids and the fungi contribute to the regulation of their host populations. These makes entomopathogenic fungi a better substitute to chemical insecticides, and pose no risk to man, domestic animals, wildlife and non-target invertebrates.

The objective of this study was to evaluate the occurrence entomopathogenic fungi in soil from different location and to test the pathogenicity of the fungus against *Aedes eagyptii* larvae under laboratory conditions.

Materials and Methods

Isolation and identification of entomopathogenic fungi

Entomopathogenic fungi was isolated from soil at different location at the Botanical garden ATBU, Bauchi. Insect baiting was conducted at three locations at distance of 15 cm between each hole. The holes were 5cm depth and 5cm in diameter. 30ml of distilled water was added to each hole and allowed to absorb for 30minute. Two insects were buried in each hole and allowed for 7days. After 7 days the insects were harvested with or without incipient visible, external fungal growth were washed with 70% ethanol and allowed to dry under sterile condition for 5 minutes. The surface of the insects was then scrapped with sterile wire loop under laminar flow hood. Serial dilutions of the three isolates were made up to five dilutions and cultured on DOA containing 200 µg/ml of dodine oatmeal agar 50 µg/ml of streptomycine (Liu *et al.,* 2007). The plates were incubated for 14 days at 25 ^{0}C. After 14 days the resulting colonies were identified macroscopically using Entomopathogenic Fungi atlas as described by Humber, (1997).

The isolates of the identified *Metarhizium anisopliae* was cultured on potato dextrose agar and incubated at 25 ^{0}C for 14 days. The fungal occurrence at the three locations was determined by the measure of the conidial formation of the isolates from each of the sites. The conidial concentration was determined by scrapping the surface of the seven days old culture and suspended in solution of 0.01% Tween20 in distilled water and stirred for 10 min. The conidial occurrence or concentration was determined by direct count using hemocytometer. Suitable isolate for the bioassay test was chosen by screening out isolates with poor conidial growth.

Mosquito's larvae rearing

The insect culture was obtained from the Zoology laboratory department of biological science ATBU, Bauchi. *Aedes aegyptii* larvae were maintained in the laboratory at room temperature 25 ^{0}C. The

larvae were maintained in a separate container containing distilled water at a pH 7.0. Each container has approximately 100 larvae. The larvae were fed with yeast powder every 24 hours.

Bioassay test

The Conidia of the isolated fungi (*Metarhizium anisopliae*) with best conidial growth was tested against *Aedes aegyptii* larvae by praying fungal suspension to a plastic container containing 50 ml of distilled water with 23 larvae. The fungal doses were made using 0.01% Tween20 to obtained $1.8.10^3$, $3.6.10^3$, and $5.4.10^3$ conidia/ml. Each container was sprayed with 10 ml of fungal suspensions ($1.8.10^3$, $3.6.10^3$, and $5.4.10^3$ conidia/ml). Control treatments were sprayed with 10 ml of distilled water. Each assay was conducted five times. Larval mortality was evaluated daily for six days.

Statistical Analysis

Data collected on fungal pathogenicity were subjected to probit analysis (Finney, 1971). Correlation between probit of mortality and log of concentration were established.

Results and Discussion

The occurrence of and *Metarhiziun anisopliae* at different soil locations and the efficacy of the fungi on *Aedes eagyptii* larvae were assessed over a period of time. The result of the study showed a variation in the occurrence of the fungi at the three different locations. Location A and B had the highest occurrence with over 70% and less than 70% conidia compared to location C with conidia formation of less than 25% as shown in (Table 1). Conidial concentration was obtained direct count using hemocytometer. This finding agrees with Benserradj *et al.*, (2014) who reported that *Metarhizium* is one of the most common entomopathogenic fungi with a worldwide distribution.

Table 1. The occurrence of *Metarhizium anisopliae* at different locations

Sampling Hole	Location A	Location B	Location C
1	+++	++	++
2	++	-	-
3	-	+++	-

Note: +++ indicates 70% conidia, ++, less than 70% conidia, +, less than 25% conidia, -, no conidia.

The effects of *Metarhizium anisopliae* on *Aedes aegyptii* larvae revealed a progressive increase in mortality with increased in time. The percentage mortality varied from 4% to 100%. Rapid Larval mortality was observed two days after praying (Figure 1). This may be as a result of the time required for the conidia to germinate and penetrates into the larvae. Mortality was highest and relatively uniform at the highest concentrations ($3.6.10^3$ and $5.4.10^3$ conidia/ml) at day 4, 5, and 6 after treatment. Maximum mortality in all the concentration occurred between one to three days. Similar observation was reported by Scholte *et al.*, (2003) that, the entomopathogenic fungi takes time to kill mosquito depending on the concentration and species. Larval mortality with different concentrations revealed increased mortality of 0% to 60%, 4% to 73% and 8% to 91% in conc. 1, ($1.8.10^3$ conidia/ml), conc. 2, ($3.6.10^3$ conidia/ml) and conc. 3, ($5.4.10^3$ conidia/ml) respectively as shown in (Table 2). This study was in accordance with McCray et al., (1973) who reported that *M. anisopliae* successfully infect and kill *Aedes aegyptii*, unlike another mosquito.

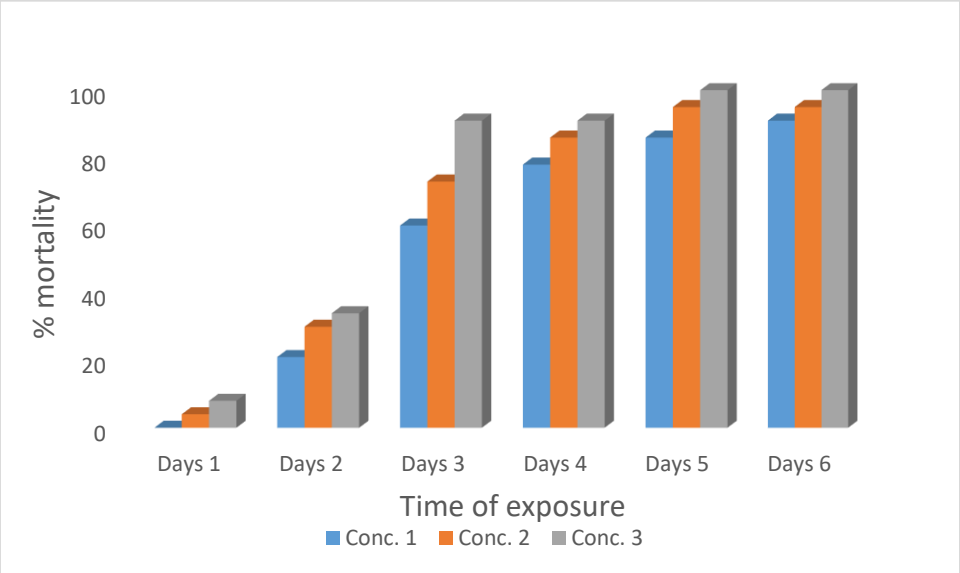

Figure 1. Graph showing percentage Larval mortality with time

The pathogenicity of *Metarhizium anisopliae* conidia on mosquito larvae expressed in term of LC_{50} and LC_{90} was observed as $14.5.10^3$ and $41.5.10^3$ conidia/ml after 24 hours, and $12.9.10^3$ and $331.1.10^3$ conidia/ml after two days (48 hours). After three days the calculated value of LC_{50} was $1.4.10^3$

conidia/ml while LC_{90} value observed was $5.4.10^3$. The calculated LC_{50} and LC_{90} values after day 4, 5 and 6 were $0.4.10^3$ and $4.4.10^3$; $0.4.10^3$ and $2.1.10^3$; and $0.1.10^3$ and $1.5.10^3$ conidia/ml respectively as shown in (Table 2). As observed in this study a number of work showed some strains of *Metarhizium* are virulent against mosquito larvae (Alves *et al*., 2002; Scholte *et al*., 2005; Amora *et al*., 2010; Benserradj *et al*., 2014).

Table 2. The pathogenicity of Metarhizium anisopliae against Aedes eagyptii larvae

	Duration of exposure					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Concentrations (conidia /ml)						
Control	0	0	0	0	0	0
$1.8.10^3$	0	21	60	78	86	91
$3.6.10^3$	4	30	73	86	95	95
$5.4.10^3$	4	34	91	91	100	100
LC_{50}	**$14.5.10^3$**	**$12.9.10^3$**	**$1.4.10^3$**	**$0.4.10^3$**	**$0.4.10^3$**	**$0.1.10^3$**
LC_{90}	**$41.5.10^3$**	**$331.1.10^3$**	**$5.4.10^3$**	**$4.4.10^3$**	**$2.1.10^3$**	**$1.5.10^3$**

The result of the study also showed a highly positive correlation between mortality and log of conidia concentrations as shown in (Figure 2)

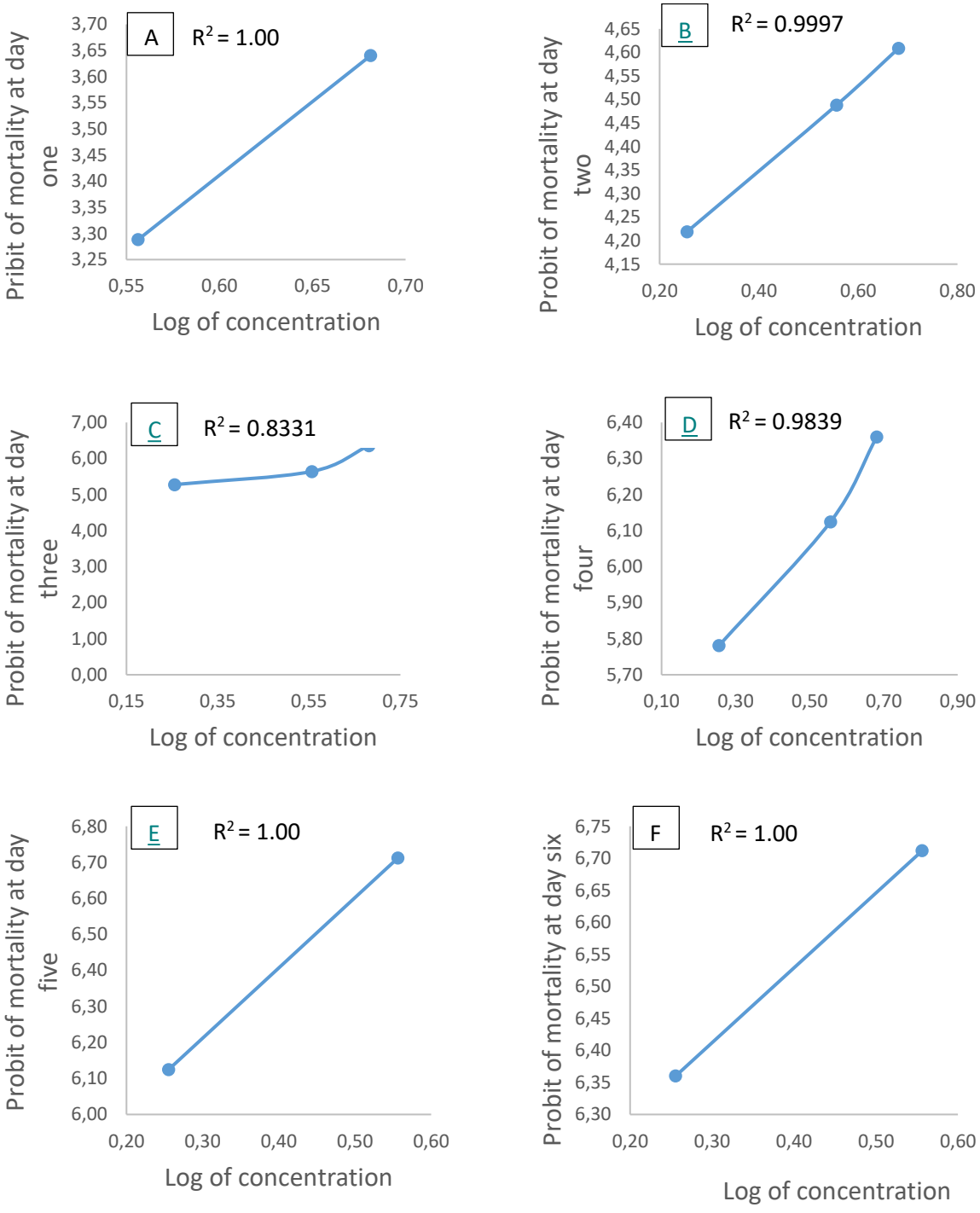

Figure 2. The relationship between probit of mortality and dose concentration with A, after 24 hours, B after two days, C, after three days, D, after four days, E, after five days and F, after six days

The findings of the present study showed the virulence of *M. anisopliae* against larvae *Aedes eagyptii*, the percentage mortality of the mosquito larvae was up to 100% and the mortality increased progressively with increase in time of exposure. Benserradj *et al*., (2014) report a mortality of 96% in *C. pipiens* larvae. Daoust and Robert (1983) reported that percentage larval mortality was enhanced significantly with increased concentration and time.

This study demonstrates that variation exists in the occurrence of entomopathogenic fungi at different soil location. Highlights the potential of using *Metarhizium anisopliae* as a biocontrol agent of mosquitoes and suggest the opportunity to explore *Metarhizium anisopliae*-mosquito interaction at the molecular level.

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