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Determination of the Efficacy of Entomopathogenic Fungus (*Metarhizium anisopliae*) for the Control of *Culex quinquefasciatus*, a Filariasis Vector

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Abstract

Several mosquito species serve as vectors for many parasitic diseases. Control measures of such vector borne diseases rely majorly on the use of chemical insecticide, which in turn has an adverse effect on both the environment and public health. There is an increase need for an eco-friendly approach to counteract the negative impact of the conventional chemical insecticide. In this study, the efficacy entomopathogenic fungi *Metarhizium anisopliae* was tested against the larvae of *Culex quinquefasciatus* under laboratory condition. Fourth instar larvae of *Culex quinquefasciatus* were treated against different concentration (10^7, 10^8, 10^9 and 10^{10} conidia/ml) of *Metarhizium anisopliae*, each treatment containing 20 larvae with three replicates and a control. The experiment was allowed to run for a period of 120 hours. Mortality was recorded at an interval of 24 hours. Probit analysis was used to determine LC_{50}, LC_{90}, LC_{95} and LC_{99}. For all the selected concentration, highest mortality of 42 (70,0%), 48 (80.0%), 51 (85.0%) and 57 (95.0%) were recorded after 120 hour. 1.79×10^3, 3.96×10^4, 4.97×10^5, 7.58×10^8 conidia/ml were the LC_{50}, LC_{90}, LC_{95} and LC_{99} recorded respectively. *Metarhizium anisopliae* has the potential to be used as biocontrol agent for *Culex quinquefasciatus* and is suitable candidate for further research.

Keywords: Biological control; Entomopathogenic fungi; *M. anisopliae*; *Culex quinquefasciatus*

Introduction

Several mosquito species are known to serve as vectors for several parasitic diseases such as malaria, filariasis, yellow fever, Japanese encephalitis, dengue, and Zika virus [1]. Malaria and filariasis are among the most common prevalent parasitic disease globally [2], as of 2011, 1.3 billion were at risk of the filariasis and also about one hundred and twenty million people were infected with disease globally [3]. Presently conventional control measures of several mosquito borne infection like malaria and lymphatic filariasis heavenly rely on vector control which in turn depends heavily on the use of chemical insecticide like parathyroid and personal preventives which involves the use of Insecticide Treated Nets (ITN) and Indoor Residual Spraying (IRS) [4,5].

The adverse consequences of synthetic chemical insecticides in the environment are a serious and major public health problem. Over the last fifty years, many problems have been resulted due to the misuse of synthetic insecticides in agriculture and public health programs, such as insecticide resistance, environmental pollution, toxic hazards to human and other non-target organisms [6], coupled with accumulation of these chemicals in the food chain and environmental pollution [7], these necessitates exploring eco-friendly and biological control methods [8]. Therefore in past few years' interest in mosquito control using entomopathogenic fungi is evident, mainly due to continuous and increasing levels of insecticide resistance and increase in global risk of mosquito borne diseases [9]. The use of entomopathogenic fungi has so many advantages over conventional insecticide and pesticide, in that, having target selectivity, environmental compatibility, economic variability, novel mode of action, safer to environment and beneficial organisms as well as rational approach at a long run [10].

Entomopathogenic fungi which are normally found in soil and are widespread in temperate agro-ecosystems and semi-natural habitats are a very heterogeneous group of insect pathogens, there nearly 700 species belonging to approximately 100 orders [11]. Entomopathogenic fungi have the ability to target the larval stage of the pest and vector right from the breeding site which is more advantageous and attractive targets for the fact that insects like mosquitoes usually breed in stagnant water and thus, it is easy to deal with them in such habitat (breeding site) [12,13]. The genus *Metarhizium* is pathogenic to a large number of insect species thus, are among the natural enemies of pests and Vectors. It causes a disease known as 'green muscardine' in insect hosts because of the green colour of its conidial cells [14]. Entomopathogenic fungal metabolites could be an alternative source for mosquito larvicides because they constitute a potential source of bioactive compounds and generally free from harmful effects [15]. For example, *M. anisopliae* has a large host-range, including arachnids and five orders of insects, comprising over 200 species. Despite the fact that, mosquitoes are not listed as natural hosts for *M. anisopliae* some strains have shown to be virulent against mosquito larvae. Spores (conidia) of *M. anisopliae* have been known for some time to be infectious to adults and emerging pupae of some insects. Therefore, the aim of the present study was to isolate and determine the efficacy of *Metarhizium anisopliae* in the control of larval stage of *Culex quinquefasciatus*.

Materials and Methods

Study area

The study was conducted at Abubakar Tafawa Balewa University Bauchi (ATBU), Yelwa Campus (Longitude 9.792° East and Latitude

10.279º North) Bauchi Local Government Area, Bauchi State, Nigeria.

Mosquito (*Culex quinquefasciatus* collection and breeding

One hundred and ninety two (192) blood fed mosquitoes were collected from their resting site in some selected houses of Gombe metropolis using aspirators. The collected mosquito species were placed in collecting cubs and transported to the insectary unit of biological sciences laboratory of Gombe state university for confirmation and identification. 109 (56.77%) were identified to be *Culex quinquefasciatus* and released in to two separate breeding cages for rearing and breeding, while in the cages they were fed with 10% sugar solution. Five (5) egg cups each containing 500 ml dechlorinated water were placed in each for eggs laying. Pieces of filter paper were placed on to the water and allowed to float for easy recovery of the eggs laid. The eggs were collected from the filter paper the following morning and transferred in to containers (45 × 20 cm) containing 500 ml of unchloronated water. No any food was provided to the containers until the first instar appears, then they were fed with 10% yeast and filtering was conducted once the water was dirty. The larvae were monitored until third and fourth instar larvae emerged, and were ready for the experimentation.

Fungal culture and conidial production

An inoculum of *Metarhizium anisopliae* was obtained from the biological science department Abubakar Tafawa Balewa University, Bauchi. Method of was adopted, but slight modification in culturing of *Metarhizium anisopliae*. In this, briefly the *Metarhizium anisopliae* were cultured on Potato Dextrose Agar (PDA) medium in an aseptic cupboard fumes and incubated at 28°C for 3 days. The conidia were harvested by scraping the surface of 3 days old culture suspended in distilled water. The mixture was stirred with a magnetic stirrer for 10 minute. The conidial concentration was determined using a hemocytometer which was used to count the number of conidia under a compound microscope. The conidial suspension was further diluted with 0.05% Tween 80 solution, until it reaches a desired concentration with a countable number of spores. After having the established concentration of conidia, suspensions were diluted with distilled water to the concentrations of 1×10^7, 10^8, 10^9, and 10^{10} conidia/ml. Only distilled water and 0.01% of poly sorbent added in the control treatment.

Bioassays

Laboratory bioassay was done following the methods of world health organisation with some modifications. Conidia of *M. anisopliae* were tested larvae of *C. quinquefasciatus* by adding fungal suspension to plastic cups containing water with 20 larvae of *C. quinquefaciatus*. Each plastic cup was inoculated with 1 ml of fungal suspensions of varying concentration (10^7, 10^8, 10^9, and 10^{10} conidia/ml). Control treatments were prepared by adding of 20 ml of distilled water and 0.01% poly sorbent only. Each assay was replicated three times. Larvae were fed with yeast powder and their mortality was observed in a 24 h interval for 5 days.

Mortality determination

A glass rod was used to determine whether the larvae are dead or not. The rod was dipped into the container and bring very close to suspected dead larvae (which usually lie flat on the water surface). For the larvae that was still alive responded rapidly by either bending or moving away from the rods. On the other hand, for the dead larvae no matter how close the rod was brought, there was no any respond.

Mycosis test

For this test, three petri dishes were set up; two containing distilled water and the other one with 70% alcohol. Firstly all dead *Culex quinquefasciatus* larvae were placed in to the distilled water one by one, later in to ethanol and then into the distilled water again in order to kill the fungus on the surface of the larvae. After which microscopic examination of treated *Culex quinquefasciatus* larvae was conducted. Any sign of fungal growth on the body surface the larvae indicated the actual number of insect that died from fungal infestation.

Statistical analysis

All data generated were entered in to an excel software, and later transferred into a Minitab software for the actual analysis. Probit regression analysis was used to determine the relationship between concentration and mortality of *Culex quinquefasciatus* in given treatment. The LC_{50}, LC_{90}, and LC_{99} values were calculated with 95% confidence limit.

Results

Result from this study shows that *M. anisopliae* isolate tested against fourth instars larvae of *C. quinquefasciatus* has pathogenic effect in all the treatment, but there was no mortality recorded in all the controls. However, pathogenicity varied according to concentration of spores and period of exposure. For the four concentrations; 10^7, 10^8, 10^9, and 10^{10} conidia/ml of the fungal isolate tested, it was observed that, mortality increased with increased in the time of exposure and also increased as the conidia concentration increased (Figure 1). Highest mortality of 57 (95%) was recorded at the highest concentration (10^{10}) of conidia/ml applied. Consequently, other lower concentrations of 10^7, 10^8 and 10^9 conidia/ml recorded a Culex larval mortality of 42 (70%), 48(80%) and 51 (85%) respectively.

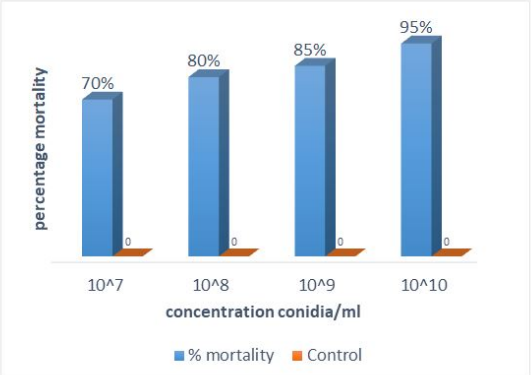

Figure 1: Total percentage mortality of *Culex* larvae exposed at different concentration of *Metarhizium anisopliae* isolate.

Mortality for the efficacy of *Metarhizium anisopliae* was assessed against *Culex quinquefasciatus* larvae based on the exposure period at various conidia concentration (10^7, 10^8 and 10^9 10^{10}conidia/ml). The

result revealed that, mortality increases as the exposure time also increases as shown in Table 1 below.

Concentration	Time/hour				
(Conidiag/ml)	24 h	48 h	72 h	96 h	120 h
	Mortality (%)	Mortality (%)	Mortality (%)	Mortality (%)	Mortality (%)
10^7	0	3 (5.0)	15 (20)	26 (43.3)	42 (70.0)
10^8	0	6 (10.0)	17 (28.3)	30 (50.0)	48 (80.0)
10^9	0	09 (15.0)	18 (30.0)	32 (53.3)	51 (85.0)
10^{10}	3 (5.0)	12 (20.0)	26 (43.3)	41 (68.33)	57 (95.0)
Control	0	0	0	0	0

Table 1: Percentage mortality (%) of mosquito larvae *Culex quinquefasciatus* exposed at different time and concentrations of *Metarhizium anisopliae.*

At the concentration of 10^7, 10^8, 10^9, in 24 hours of exposure time there was no any mortality but at 1010 concentration 3 (5%) larval mortality was recorded. After 48hours of post exposure, mortality increases to 3 (5%), 6 (10), 9 (15) and 12 (20%) at 10^7, 10^8, 10^9, 10^{10} Conidiag/ml respectively. 15 (20), 17 (28.3), 18 (30) and 26 (43.3%) mortality were observed after 72 hours of exposure time using 10^7, 10^8, 10^9, 10^{10} Conidiag/ml. 26 (43.3), 30 (50), 32 (53.3) and 41 (68.33%) after 96 hours of exposure to the concentration of 10^7, 10^8, 10^9, 10^{10} Conidiag/ml while at 120 hours of exposure to the same concentration 42 (70), 48 (80), 51 (85) and 57 (95%) mortality were respectively recorded.

The survival data of biological vector, *Culex quinquefasciatus*, closely fitted the probit distribution model which showed a significant increase in the mortality rate of *C. quinquefasciatus* following exposure to different doses of conidia. It can be indicated from this result that survival rates were inversely related to the exposure of dose. That is, the higher the dose of conidia to the test organism, the increase in the mortality rate, While the lowest mortality rate was observed in the lowest concentration of conidia (10^7) and the higher mortality rate was found in the higher concentration of conidia (10^{10}), as shown in Figure 2 below.

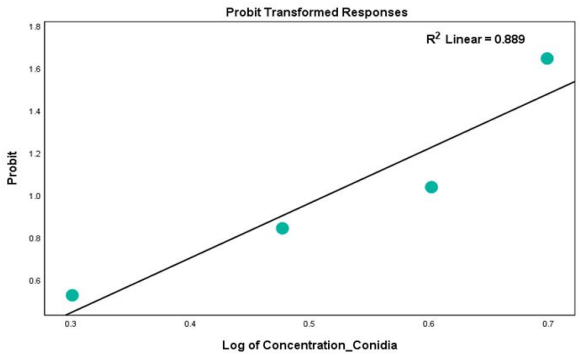

Figure 2: Probit survival curve for larva of *Culex quinquefasciatus* infected with different doses of conidia of the entomopathogenic fungus *Metarhizium anisopliae*.

The result of the probit modelling analysis (Table 2) revealed the lethal concentrations that cause 50% (LC_{50}), 90% (LC_{90}), 95% (LC_{95}) and 99% (LC_{99}) mortality of *Culex quinquefasciatus* exposed to different doses of conidia of the entomopathogenic fungus *Metarhizium anisopliae* as shown in Table 3, 1.79×10^3 conidia/ml (95% CI was 0.23 to 2.54), 3.96×10^4 conidia/ml (95% CI was 2.80 to 25.51), 4.97×10^5 conidia/ml (95% CI was 3.37 to 82.92), 7.58×10^8 conidia/ml (95% CI was 4.46 to 810.92) were reported as the LC_{50}, LC_{90}, LC_{95} and LC_{99} respectively.

LC	Estimate	Lower bound	Upper bound
LC_{50}	1.79	0.23	2.54
LC_{90}	3.96	2.8	25.51
LC_{95}	4.97	3.37	82.92
LC_{99}	7.58	4.46	810.92

Table 2: Estimation of Lethal Concentrations (LC) of *Culex quinquefasciatus* exposed to different concentration of conidia.

Specie	Response	R^2	Pearson Goodness of fit *Chi square*	Sig. level	95% confidence interval

					Lower bound	Upper bound
Culex quinquefasciatus	y=3.71 + 0.52x	0.89	10.17	0.02*	2.69	4.72
*Represent significant (P < 0.05). Since the significance level is less than 0.05, there is significance between the observed and the expected value.						

Table 3: Probit transformed response for Culex quinquefasciatus following exposure to varying doses of conidia of Metarhizium anisopliae.

Mycosis results showed that the hypae of the fungal conidia start to appear after 172 hours post treatment with 25% *C. quinquefasciatus*. Highest (91%) conidial appearance was observed at 244 hours of post treatment. Conidial appearance on the surface of 67% and 50% for mosquito larvae after 220 and 196 hours of post treatment respectively. There was no conidial growth on the surface of *C. quinquefasciatus* after 148 hours of post treatment as shown in Figure 3 below.

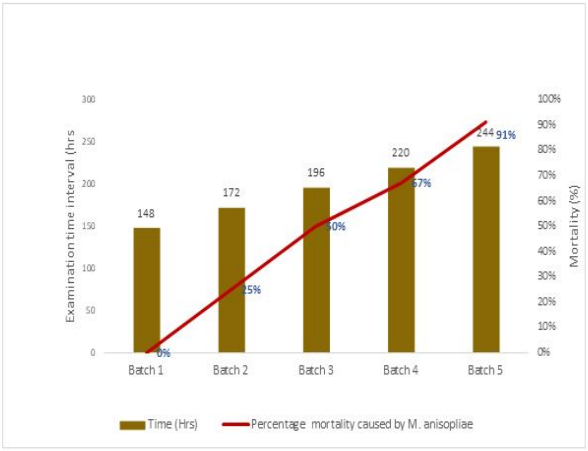

Figure 3: percentage mortality of *Culex quinquefasciatus* caused by *Metarhizium anisopliae* during five batches examination of cadavers under microscope.

Discussion

The present study evaluates the bio-efficacy of the fungus, *M. anisopliae* against larval stage of *C. quinquefasciatus* under laboratory conditions. The findings from the study revealed a very promising outcome, where *M. anisopliae* demonstrated high level of pathogenic activity against *C. quinquefasciatus* larva. This is not surprising, as previous studies revealed that Spores and metabolites of entomopathogenic fungi have been reported as larvicides/biocontrol agents against larval stage of different mosquitoes species [16], coupled with the fact that *M. anisopliae* is among the best natural enemy of several biological vector [17]. Therefore *M. anisopliae* could serve as a better replacement present chemical insecticide and pesticide in a future. Findings from this study are in agreement to the findings of who reported that, *M. anisopliae* causes significant mortality in different mosquito including *C. quinquefasciatus* [18]. The larvicidal activity of *M. anisopliae* is dose dependent, this findings is in concordance with findings who equally reported positive correlation between larval mortality and concentration of the entomopathogenic fungi (*M. anisopliae*), for that high level of larval mortalaity of 95% was reported from 1×10^{10} conidia/ml which was the highest concentration [19,20]. This result is the very similar to the findings of who equally reported reported high larval mortality of 91% at a conidial concentration of 1×10^{8} Conidia/ml [21]. This clearly demonstrates the impact of concentration on larvicidal actitivity of the Entomopathogenic fungi. In addition, larvicidal activity of *M. anisopliae* against *C. quinquefasciatus* is directly proportional to the time of exposure that is larval mortality increases as sharply from 24hours up to 120hours, this finding is in agreement with findings of who also reported similar condition [22]. Therefore the longer the exposure time of lavae to the *M. anisopliae* isolate more the metabolite and other active component and toxins such as Destruxin, Bavericin, and Efrapeptins are secreted by *M. anisopliae* thereby penetrate deeply and establish themselves and eventually kill the larvae [23].

Irrespective of time, the lethal concentration that will kill at least 50% (LC_{50}) recorded in this study was 1.79×10^{3} conidia/ml with the range of 0.23-2.54 conidia/ml. Considering this low value of LC_{50} and a very lower boundary range of 0.23 confirmed the effectiveness of *M. anisopliae* as good biological control agent. This result is by far lower than 3.9 x 10^{8} reported as LC_{50} conidia/ml of *Metarhizium anisopliae* against mosquito larvae after 24 by [24]. Mycosis result of this study confirmed the actual effectiveness of *M. anisopliae* as more 90% of the larval mortality in was attributed to *M. anisopliae* isolate due to the development hypae of the fungal conidia on the dead *C. quinquefasciatus* larvae 244hours after treatment.

Conclusion

M. anisopliae significantly demonstrate high level of efficacy in the control of larval stage of *C. quinquefasciatus*, as 95% of larval mortality was reported at higher conidia/ml concentration of 1×10^{10}. Likewise other lower concentration demonstrates relatively good larvicidal activity even at 24 hour of larval exposure. Therefore in a near future entomopathogenic can favourably compete and even replace conventional synthetic insecticide.

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