



Larvicidal Efficacy of Entomopathogenic Fungi Isolated from the Farmland Areas in Osogbo South-West Nigeria

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Abstract

Thousands of entomopathogenic fungi are in existence, unexploited and it is important to identify and use for biocontrol of mosquito larva. The aim of this study was to determine the larvicidal efficacy of newly identified entomopathogenic fungi. Soil samples were taken from three different locations in a farmland at Osun State University (UNIOSUN), Osogbo, Nigeria for isolation and identification of entomopathogenic fungi and test its larvicidal effect on mosquito larvae. The samples were analyzed using spread plate technique which include serial dilution of samples, inoculation on prepared Sabouraud Dextrose Agar using three dilutions (10^{-1} , 10^{-3} , 10^{-10}) and incubation at 27°C for 72 hours after which the plates were examined for growth followed by sub-culturing. Mosquito larvae were bred, picked into sterile distilled water in Petri dishes. The spores of the fungi were scraped on the larva to determine the larvicidal effect of the fungal isolates at varied interval: 40 minutes, 2 hours, 24 hours, and 48 hours. A total of seven fungi were isolated two (*Aspergillus flavus* and *Aspergillus niger*) of which were able to show larvicidal effect on mosquito larvae. Some larvae died at exactly 40 minutes of inoculation when tested with *Aspergillus niger*, some took hours (48 hours) while others remain viable. This study concludes that *Aspergillus niger* and *Aspergillus flavus* isolated from soil samples exhibited biolarvicidal efficacy against mosquito larvae and the mortality rate increases with time of exposure. Entomopathogenic fungi should therefore be explored for control of mosquitoes in our environment.

Introduction

Mosquitoes are well known vector of human and animal pathogens and millions of people are killed

by mosquito-borne diseases every year such as malaria dengue, chikungunya, Zika, yellow fever, encephalitis and filariasis.¹ Vector control,

sanitation, habitat disruption or personal protection from mosquito bites are the most widely measures employed to control and protect people from infection of these diseases.² Over the past few decades, many countries organized official programs of mosquito vector control. Currently, synthetic chemical pesticides against adults or larva have been the mainstay and are the most widely used for control of Mosquitoes. Mosquito larvae are the attractive targets for these pesticides because mosquitoes breed in water and thus, it is easy to deal with them in this habitat³ The indiscriminate use of chemical insecticides to target adult mosquitoes has given rise to problems such as mosquito resistance, environmental contamination, and health risk to humans and non-target organisms. To mitigate these problems, there is an urgent need to develop alternatives to conventional chemical insecticides, which are safe, effective, biodegradable and highly selective. There has been an increasing awareness in the possibility of using biological control agents as alternative to chemical control of mosquitoes. Among the eminent biological control agents are entomopathogenic microorganisms such as fungi as larvicides.⁴ Fungal biocontrol agents are the most important among all the Entomopathogenic microorganisms due to easy delivery, chances to improve formulation, vast number of pathogenic strains known, easy engineering techniques and its ability to control both sap sucking pests such as mosquito and aphids as well as pest with chewing mouth parts. They include numerous phylogenetically, morphologically and ecologically diverse fungal species which evolve to exploit insects with their main route of entry being through the insect's integument, by ingestion or via wounds or trachea.⁵ Most entomopathogenic fungi can be grown on artificial media (shin *et al.*, 2009); being natural mortality agents which are environmentally safe, there is a worldwide interest in the use and manipulation of entomopathogenic fungi for biological control of insects and other arthropod pests.⁶ They display a higher degree of effectiveness in infecting their

host, acting as regulators for numerous harmful insects including both domestic and forest insects.⁷

In general mosquitoes show susceptibility towards entomopathogenic fungi and its derived products. They have low toxicity to non-target organisms and using entomopathogenic fungi as larvicides may be a promising approach for biological control of mosquitoes due to their selective toxicity and ready decomposability in the ecosystem.⁸ Also, unlike the inherent dangers which are associated with the process of production of synthetic insecticides, the process for the manufacture of microbial products is safe and less pollutant.⁹

This study therefore aimed at determining the larvicidal activities of fungal isolates with a view to exploring their potential for biological and environmental control of mosquito-borne diseases.

Materials and Methods

Collection of Soil Samples

The study area was a farmland within Osun State University, Osogbo, Nigeria. About 100g of soil samples from 10 – 15cm deep were aseptically collected with sterilized hand trowel into sterile Aluminum foil paper from three different locations on the same farmland and transported to the laboratory for further analysis.

Isolation of Fungi in the Soil Samples

Fungi were isolated from soil samples using serial dilution method; one gram (1g) of soil sample was suspended in 9ml of sterile diluents and serially diluted up to tenth factor (10^{-10}). Dilutions of 10^1 , 10^5 and 10^{10} was used to isolate the fungi. With sterile syringe, 0.1ml of each dilution was drawn and inoculated into the plates which have been labeled and arranged according to dilution, then glass spreader dipped into 100% ethanol and flamed was used to spread the inoculums in the petri dish by slightly opened beside the flame to avoid contamination. All plates were incubated at room temperature ($25^{\circ}\text{C} - 27^{\circ}\text{C}$) and observed for growth between 3 to 5 days i.e. (72 hours – 120 hours). The process was carried out in duplicate.

Sub culturing of Fungal Isolates

Distinct fungal colonies were aseptically picked from the mixed culture using sterile inoculating needle and transferred onto a freshly prepared sterile SDA plates and incubated for 3 – 5 days at 25°C. This process was repeated until pure cultures were obtained pure culture was maintained on SDA slant and stored at 4°C in the refrigerator for further analysis.

Macroscopic and Microscopic Identification of Isolated Fungi

Each pure culture plate was observed for morphology characteristics of the isolates including color, growth rate and texture.

For presumptive identification of isolated fungi from soil samples, a drop of lactophenol blue was placed on a clean glass slide, and an inoculating needle was used to pick from the pure culture plate onto the stain on glass slide. The cover slip was gently placed on the prepared smear, mounted and view under X40 objective on Microscope. The Microscopic appearance was captured and compared with the structures in soil fungi Compendium.¹⁰

Breeding of Mosquito Larvae

Buckets containing half-filled dechlorinated water were placed under shade at different site locations for 7 days around the school General Laboratory of the institution. After breeding, the buckets were transported into the laboratory.

Rejuvenating of the Isolates

Each fungal isolate was taken from the stock culture and inoculated on freshly prepared sterilized Saboraud Dextrose agar plates, incubated at room temperature (25° – 27°C) for 72 hours.

Procedure of Biolarvicidal Efficacy

One milliliter of sterile distilled water was dispensed into each petri dish onto which the spores of fungal spores were scraped and the concentration standardized and determined by measuring its optical density (turbidity) (labeled as higher concentration) by previously documented protocols,^{11,12} then four larvae were

picked and transferred into the spore suspension in the petri dishes. A lower spore concentration was prepared by further diluting 1ml of the standard concentration (prepared as above) with 1ml of distilled water to decrease the concentration to half (labeled as lower concentration), the same quantity of larvae (four larvae) were then introduced into this spore suspension. Control treatment contains sterile distilled water and mosquito larvae in a Petri dish. Death or viability of mosquito larvae was monitored and recorded at room temperature (25°C – 27°C) from the point of inoculation to three days.

Confirmation Test

To confirm that the fungal isolates were responsible for the mortality of mosquito larvae, the dead larvae were picked and inoculated on freshly prepared Saboraud Dextrose Agar. The growth of the fungal isolates was shown on the plates after 72hrs.

Results

Fungi species Isolated from Soil Samples

A total of seven (7) fungi were isolated from the soil samples and they included *Microsporium* spp., *Fusarium* spp., *Malbranchea entero arthric*, *Aspergillus niger*, *Aspergillus flavus*, *Onychocola Canadensis*, and *Arthrinium* spp.

Table 1 and Figure 1a and 1b show the morphological characteristics and microscopic appearances of the isolates through direct observation of colonies on solid media plates as well as viewing under x40 objectives.

Result of Biolarvicidal Effect of Fungi

Biolarvicidal potential of isolated fungi was carried out and it revealed that *Aspergillus niger*, *Aspergillus flavus* have efficacy against the mosquito larvae, but the rate at which death occur in each isolate is different. Result is shown in Table 2 and 3.

Confirmation of Biolarvicidal Effect of Fungi

This shows that the fungi isolates were responsible for the mortality of mosquito larvae

by observing growth of fungi on solid media plates after incubation.

Mortality Rate of Mosquito Larvae after Treatment with Fungi

Table 2 shows that isolate A-1a (*Microsporium* spp.) had no larvicidal effect on the all the four larvae treated at lower spore concentration (25.25mg/L), the same effect was observed for isolates A-1b (*Fusarium* spp.), B-1c (*Malbranchea entero arthric*), C-5a (*Onychocola canadensis*) and C-5c (*Arthrinium* spp.) at the same spore concentration.




However, at the same spore concentration, isolate B-5c (*Aspergillus niger*) had no effect at 2hours on the four larvae but some effect at 24hours in which two larvae were viable while two were dead (50% mortality rate). At 48 hours all the four larvae were dead (100% mortality rate). Likewise,

isolate B-5d (*Aspergillus flavus*) shows no mortality at 2hours but some effect at 24hours in which two larvae were viable while two were dead (50% mortality rate). At 48 hours all the four larvae were dead (100% mortality rate).

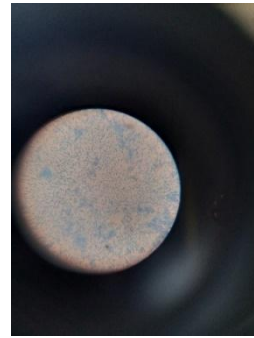
Table 3 shows mortality rates at a higher spore concentration (50.5mg/L). *Aspergillus niger* showed 100% mortality at different time of exposure ranging from 40 minutes to 24hours. This implies that *Aspergillus niger* has high larvicidal potency regardless of time of exposure. On the other hand, at the same spore concentration, *Aspergillus flavus* showed no larvicidal effect on the mosquito larvae at 40 minutes, 50% mortality at 2hours and 100% mortality at 24hours implying that the mortality rate with *Aspergillus flavus* increases with time of exposure as represented in Figure 3.

Table 1: Cultural morphology and characteristics of the isolates

SAMPLE CODE	SURFACE/REVERSE	GROWTH RATE	TEXTURE
A-1a	White wooly surface/rusty brown at the reverse	Grow slowly	Velvety to wooly
A-1b	Whitish and brown at the reverse	Rapid growth	Wooly to cottony
B-1c	White wooly	Moderately wooly	Wooly
B-5c	Black surface, yellowish filament and reverse	Rapid growth	Powdery
B-5d	Green with whitish wooly growth/yellowish at the base	Rapid growth	Powdery and wooly
C-5a	White becoming yellowish/pale	Moderately rapid	Smooth to slightly floccose
C-5c	White wooly surface and brown at the reverse	Moderate growth	Wooly to cottony

Isolate code	Surface picture	Reverse picture	Microscopic view	Presumptive identification
A-1a				<i>Microsporium</i> spp.

A-1b



Fusarium spp.

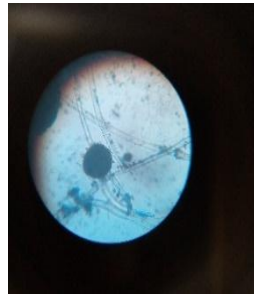
B-1c



Malbranchea spp.

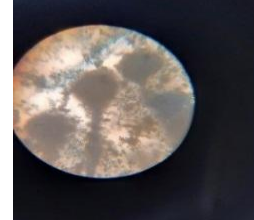
Figure 1a: Macroscopic and microscopic appearances of the isolates

B-5c



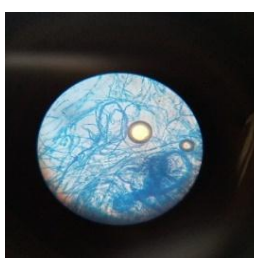
Aspergillus niger

B-5d



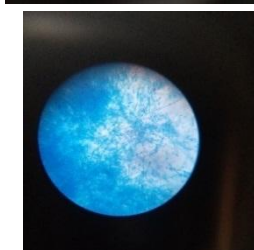
Aspergillus flavus

C-5a



Onychocola canadensis

C-5c



Arthrinium spp.

Figure 1b: Macroscopic and microscopic appearances of the isolates

Table 2: Biolarvicidal efficacy of lower concentration of fungi isolates on Mosquito larvae

ISOLATE CODE	NUMBER OF LARVAE IN 2MLS OF LOWER CONCENTRATION FUNGAL SUSPENSION	PERIOD OF INNOCULATION		
		2 hrs.	24 hrs.	48 hrs.
A- 1a	4	4V	4V	4V
A-1b	4	4V	4V	4V
B-1c	4	4V	4V	4V
B-5c	4	4V	2D 2V	4D
B-5d	4	4V	2D 2V	4D
C-5a	4	4V	4V	4V
C-5c	4	4V	4V	4V

V= Viable

D=Dead

Table 3 Biolarvicidal efficacy of higher concentration of fungi Isolates on Mosquito larvae

ISOLATE CODE	NUMBER OF LARVAE IN 2MLS OF HIGHER CONCENTRATION OF FUNGAL SUSPENSION	PERIOD OF INNOCULATION		
		40 minutes	2hrs	24hrs
A- 1a	4	4V	4V	4V
A-1b	4	4V	4V	4V
B-1c	4	4V	4V	4V
B-5c	4	4D	4D	4D
B-5d	4	4V	2D 2V	4D
C-5a	4	4V	4V	4V
C-5c	4	V	V	V

V= Viable

D=Dead

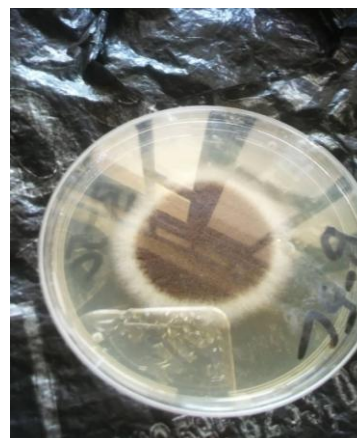
Sample Code

Name of the Isolates

Picture of the Isolates

B – 5c

Aspergillus niger



B – 5d

Aspergillus flavus



Figure 2: Confirmation test result showing growth of mosquito larvae on solid media

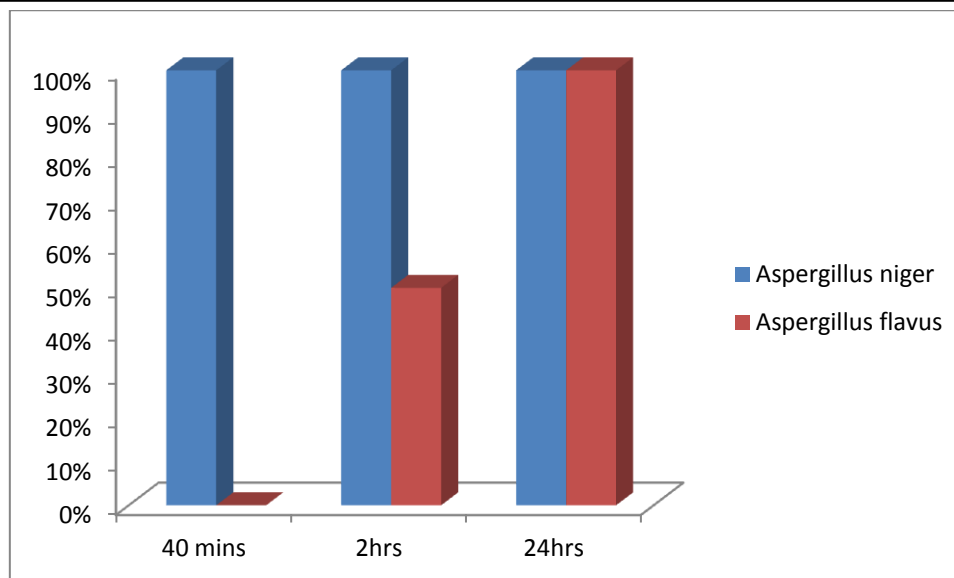


Figure 3: A chart showing the mortality rate of mosquito larvae at a concentration of 2 larvae per milliliter (4 per 2 millilitres) at different durations

Discussion

The researches on the larvicidal potential of microbes particularly fungi for controlling the mosquito larvae have only been extensively carried out on adult mosquito for several decades. Entomogenous fungi have caused high mortalities in mosquito population in many laboratories including *Aedes* and *Anopheles* species.¹³ However, a review of studies by elucidates the larvicidal potentials of fungi and revealed that the fungi could successfully infect and kill larvae of a wide range of mosquito genera with varying rates of mortality.⁶ Furthermore, a research conducted in East Africa to determine the pathogenicity of entomopathogenic fungi against several strains of adult *Anopheles gambiae* revealed a high infection rates ranging from 46 to 88% with *Metarhizium anisopliae* being the most pathogenic strain.¹⁴ In recent years, there is a considerable amount of attention focusing on identifying potential mosquitocidal fungus from natural sources for effective control of mosquitoes as a key measure to curtailing the vector borne diseases in human. A study done in Asia researching the larvicidal potential of *Lagenidium giganteum*, a water weed, noted its efficacies in killing the tested vectors with appreciable safety to non-target organisms and good biological stability.¹⁵ According to a

large-scale field trial conducted in the United States, mycelium of *Lagenidium giganteum* caused 40-90% infection rates in *Culex tarsalis* and *Anopheles freeborni sentinel* larvae.¹⁶

The potentials of many fungi have been established for mosquito control, nevertheless, only a few have received commercial attention and are marketed for use in vector control programmes globally. It is therefore becoming obvious that entomopathogenic fungi promises a greater roles in expansion of the limited options available for effective mosquito control, thereby contributing in a substantial and sustainable manner to prevention and control of vector-borne diseases.

In this study, seven fungi were isolated from soil samples and been identified as: *Microsporium spp.*, *Fusarium spp.*, *Malbranchea entero arthric*, *Aspergillus niger*, *Aspergillus flavus*, *Onychocola canadensis*, *Arthrinium spp.* The larvicidal potential of these fungi were tested at different concentration against mosquito larvae at different period of exposure (40 minutes, 2hours, 24hours and 48hours). Two isolates which were *Aspergillus niger*, and *Aspergillus flavus* exhibited larvicidal potential against mosquito larvae while other five isolates showed no effect on the larvae. At a lower fungal spore

concentration (25.25mg/L), *Aspergillus niger* showed no mortality at 2hours, 50% mortality at 24hours and 100% mortality at 48hours. At the same spore concentration, *Aspergillus flavus* showed similar mortality rates at the same time of exposure as *Aspergillus niger*. At higher spore concentration (50.5mg/L), *Aspergillus niger* showed a mortality rate of 100% mortality at 40 minutes, 2hours and 48hours. This is similar to the study by Maurya *et al.* who evaluated larvicidal potential of *Aspergillus niger*, *Aspergillus sflavus*, *Aspergillus parasiticus* against *Anopheles stephensi* and *Culex quinque fascistus* with *Aspergillus flavus* showing greatest bio efficacy at 50% lethal concentration (LC50) against larvae of *Anopheles stephensi* and *Culex quinquefasciatus* at 24 hours of exposure and bio efficacy increased in both larval species at 48 hours.¹⁷

At the same higher concentration, *Aspergillus flavus* showed no mortality at 40 minutes, 50% mortality rate at 2hours and 100% mortality rates at 24 hours which also similar to the research of Govindraja *et al* who evaluated larvicidal effect of *Aspergillus flavus* and other fungi against *Culex quinque fascistus* with high mortality rate.¹⁸ Similarly, a study conducted in northern Nigerian showed that the fungal concentration required to kill 50% of larvae decreased as the exposure duration (in hours) increased. It also reported that larval mortality rates increased proportionately with concentration of pathogenic fungi.⁸ According to a study by Bukari *et al.* fungal spores of entomopathogenic fungi, especially when formulated in synthetic oil as an effective carrier, provided a better laticidal effect with significant reduction of vector by up to 50% in Kenya, emphasizing its promising role for controlling larval populations of mosquitoes.⁹

The usefulness of fungal biolavical effects for potentiating the synthetic (chemical) laticides, even at low (environment-friendly) concentrations, had recently been studied. An Indian study demonstrated the synergistic potential of *Aspergillus flavus* and Temephos (an organophosphate laticide) by showing dramatic

reduction in malaria transmission due to enhanced larval mortality and promoted good larval control at more moderate levels of coverage even in areas where insecticide resistance makes control ineffective.⁴

Conclusion and Recommendations

From the result of this study, *Aspergillus niger* and *Aspergillus flavus* isolated from soil samples exhibited biolarvicidal efficacy against mosquito larvae. It was also shown that the mortality rate increases with time of exposure. These isolates are therefore potential be deployed for mosquito control programs singularly or in combination with other control agents. Further studies on larvicidal activity of fungi species should be carried out in this environment for evidence-based information to further establish the usefulness of these species as myco-insecticides to combat mosquito and other arthropods which are causative agents of human diseases.

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Competing interests; The authors declare that they have no competing interests.

Authors' contributions; BJA and AAW conceived and designed the study. BJA and AAW conducted laboratory experiments. ATA, OHE, BAO and ATA wrote the initial draft, ATA, OHE, BAO and ATA edited and reviewed the final manuscript. All authors approved the final report.

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