Effect of entomopathogenic fungi *Metarhizium anisopliae* var acridum and *Beauveria bassiana* on survival of *Anopheles arabiensis* Patton and *Culex quinquefasciatus* Say mosquito larvae.

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Abstract
Because of environmental considerations and the development of resistance in vectors, efforts had been directed to microbial control. Immature stages of mosquito are useful components of malaria control program. The entomopathogenic hyphomycetous fungi *Metarhizium anisopliae* var acridum and *Beauveria bassiana* were selected and tested against early (L_{1-2}) and late (L_{3-4}) stages of mosquito larvae (*Anopheles arabiensis* and *Culex quinquefasciatus*). Two concentrations of each fungual spores were used to perform the laboratory bioassays and time to death was determined for each mosquito species. *Metarhizium anisopliae* var acridum and *Beauveria bassiana* caused high mortality in *An. arabiensis* and *C. quinquefasciatus*. However, *B. bassiana* was less effective compared to *M. anisopliae* var acridum. Both *An. arabiensis* and *C. quinquefasciatus* were susceptible to the two fungi concentrations at the early stage. On the other hand, the older one were less susceptible.This research showed the potentiality of the two fungal species against mosquitoes in the larval stage, and the mortality percentage depends on fungus species itself and larval stage targeted. The increase in fungus concentration did not showed a proportional increase in mortality.

Keywords : Moequito biocontrol, Entomopathogenic Fungi, *Metarhizium anisopliae*, *Beauveria bassiana*

1. Introduction:

The development of resistance to chemical insecticides and concerns over the deleterious effects of chemical on the environment and human safety have provided a strong impetus for the development of microbial control agents for use in integrated control of insect pests. A diverse assemblage of the microorganisms are currently under consideration as insect

control agents, including viruses, bacteria, protozoa and fungi. Of fungi, a considerable effort has been focused on the development and utilization of entomopathogenic Hyphomycetes (Butt, 2001). Control measures directed against the larval and other immature stages of mosquito vectors are useful components of malaria control programs in areas where mosquito breeding sites are accessible and relatively limited in number and size (Killeen *et al*., 2002).

The entomopathogenic Hyphomycete are anamorphic fungi which reproduce by conidospores (de Hoog, 2000). The Hyphomycetes, Metarhizium anisopliae was identified more than 100 years ago, while Beauveria bassiana was noted even earlier in 1835 (Lord, 2005). The spores of these fungi have proven effective against mosquito larvae of the genus *Aedes*, *Culex* and *Anopheles* in the laboratory (Sandhu, 1993, Alves, 2000).

Unlike other biopesticides such as bacteria and viruses, entomopathogenic fungi do not need to be ingested by the larvae to cause infection, making them valuable as biological control agents. Although some reports suggest a mode of infection through the siphon tips or gut of insect larvae (Lacey *et al*., 1988), entomopathogenic fungi generally infect or penetrate their targets percutaneously (Charnley, 1989). This can occur by adhesion of spores to the insect cuticle, especially the intersegmental folds, or by simple tarsal contact (St.Leger *et al*., 1986; Scholte *et al*., 2003a). The Cuticle thickness is likely to have some effect on the rate of penetration of fungal conidia and therefore represent the first barrier of defense for the host (St.Leger *et al*., 1991; Clarkson and Charnley, 1996). Once inside the haemocoel the fungus proliferates and rapidly spreads throughout the body by means of the insect's open circulatory system. Hayphal bodies fill the haemocoel at the time of insect death with tissues being invaded and degraded to varying degrees. Hyphae eventually emerge from the cadaver producing aerial spores capable of initiating the cycle once again (Zacharuk, 1981). Figure (1) summarizes the general mode of action of fungi which develop to at least six identifiable stages from the initial infection till the death of the host which occurs between three and seventeen days post infection, depending on the species and size (Scholte *et al*., 2004a).

Use of fungal spores as a larvicide could complement adult control but in areas where the breeding site are well defined and are not being used for domestic purposes(Bukhari, 2010). A number of factors can influence larval mortality caused by fungus, e.g. species and larval stages of mosquito targeted ,the species isolate and amount of the fungus applied. In this laboratory – based study, the effect of the above mentioned factors was evaluated. Two entomopathogenic fungi *Metarhizium anispliae* var *acridium* and *Beauveria bassiana*, were tested against *Anopheles*

arabiensis, the main malaria vector in the Sudan, that breeds predominantly in clean non-polluted water. And *Culex quinquefascitus*, which breeds in a variety of habitats closely linked to human environment, usually containing highly polluted water rich in organic matter (Mboera, 1999).

The objectives:

The main objective of this study was to compare the toxicity of fungal conidia of *Metarhizium anisopliae* var. acridum and *Beauveria bassiana* against mosquito larvae, *Anopheles arabiensis* and *Culex*

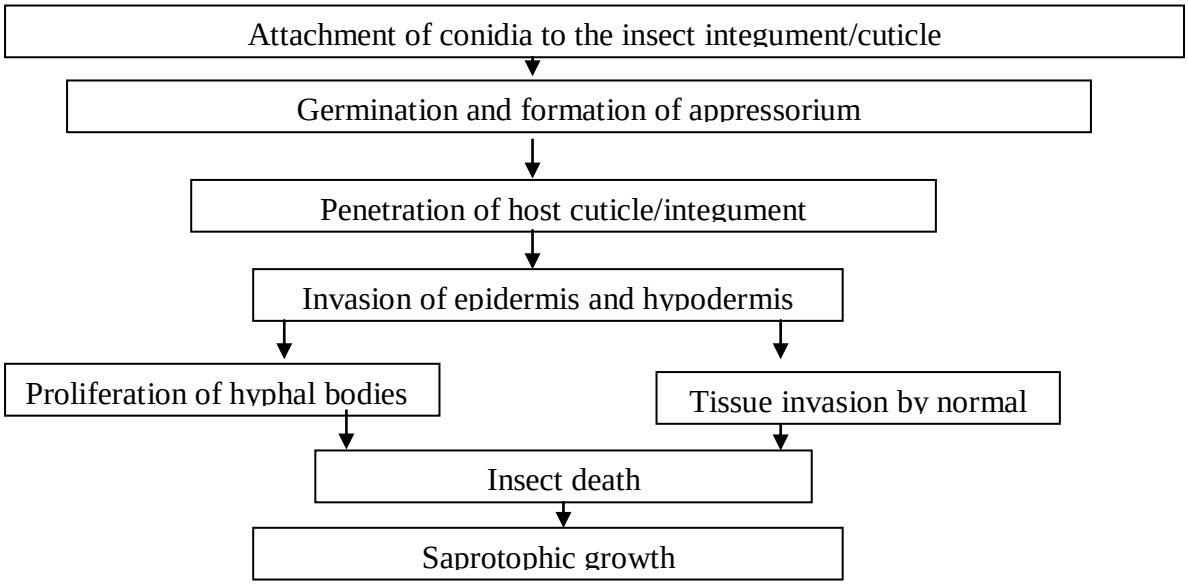

Figure (1): Schematic steps of the in vivo developmental cycle of fungal pathogens inside the insect host (Clarkson and Charnley, 1996; Narayana, 2004).

2. Materials and Methods:

2.1 Mosquito culturing:

Anopheles arabiensis colonies used in this study, were cultured in Medical Entomology Insectory at the National Public Health Laboratory (NPHL) Khartoum. All laboratory colonies were reared at 27℃ ± 2℃ and 70% ±5% relative humidity with 12/12 hours light/dark cycle. Larvae were fed on fish food tetramin. Adult females were offered cow blood twice a week. Eggs were

collected 2-3 days following blood feeding and were transferred to 25 x 8 cm hollow wire metal troughs containing distilled water for hatching. Third instar larvae were transferred to another metal trough covered with gauze in which they pupated and emerged as adults. Adults were transferred to 30 x30 x 30 cm plastic cages and were provided with a 10% sucrose solution.

Culex quinquefasciatus eggs were collected from The Sudan Natural Museum, each egg raft was transfered to 25 x 8 cm hollow ware metal troughs containing distilled water for hatching. Larvae were fed on fish food tetramin.

Entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*:

Metarhizium anisopliae var. acridum IMI 330189 dry conidia were obtained from the International Center for Insect Pathology and Ecology (ICIPE) port Sudan.

Beauveria bassiana ITCC No.6645 was obtained from the National Center for Research, Ministry of Higher Education and Scientific Research.

M.anisopliae dry conidia were used directly after a test of viability. *B. bassiana* were mass cultured to obtain enough weight of conidia and also to examine their viability before use

2.2 *Beauveria bassiana* culturing:

Beauveria bassiana was cultured in Sabouraud Dextrose Agar (SDA). Firstly the media was prepared in flasks and sterilized for 20 min at 121℃ then was poured in sterile Petri dishes and allowed to solidify. Fungal spores were taken from the original stock and inoculated onto SDA using a wire loop and incubated at 27℃ ± 1℃ in complete darkness. After two weeks (Figure 2) the conidia was harvested using a wire loop.

2.3 Mass culturing and harvest protocol of the fungus:

The entomopathogenic fungus, *beauveria bassiana* ITCC No. 6645 was mass cultured on *Sorghum* grains (Figure 3) Two hundred grams of *Sorghum* grains were washed and half-cooked by boiling in conical flasks, then autoclaved according to a standard protocol (20 min at 121℃). The material was left for 24 hours before inoculation. Loop-full of fungus, from 15 days-old culture was inoculated in each flask then the flask was plugged with cotton , kept for one month at temperature 27℃± 1℃ and 65% ± 5% relative humidity and complete darkness. Afterwards the conidia were washed off with 0.05% Triton X 100 under laminar flow. Then kept in 10 ml centrifuge tubes and centrifuged for 10 min at 6000 RPM. The collected conidia were kept at -20℃, after that they were lypholyzed for 3 days (each day 8 hours) till a fine powder was obtained (Figure 4).

2.4 Preparation of conidial suspension:

The harvested conidia from a 15 day old Petri dishes were suspended in sterile distilled water containing 0.05% Triton X 100 in bottles. The bottles were shaken vigorously until a homogeneous conidial suspension was obtained.

2.5 Germination test of fungi:

Viability of each fungus was determined by spread-plating. 0.1 ml of product's suspension was spread on SAD plates, with three replications. Then incubated in complete darkness at 27℃ ± 1℃ and relative humidity 65 % ± 5% for 24 hours then, using cork borer, round pieces of media were cut, kept in cavity slides and covered with cover slips then examined under 40 X 10 (Figure 5). Conidia were deemed to have germinated if the germination tubes were longer than half the size of the conidia.

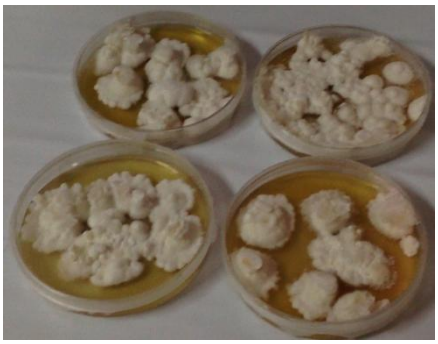

Figure 2: *Beauveria bassiana* No. 6645 cultured for 2 weeks on Sabourud Dexstrose Agar (SDA)

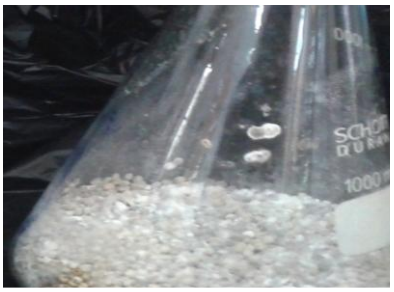

Figure 3: *Beauveria bassiana* mass cultured on Sorghum grains

Figure 4: Dry comidia after lyophilisation

All experiment were done in Medical Entomology Insectory at the National Public Health Laboratory. The climatic conditions of the test room were similar to those of the rearing room.

2.6 Pre-experimental test:

To select a suitable concentration of each fungus 5, 10, 15 and 20mg were used in a pre-test following the same procedure described below in experiment 1, using the late stage larvae (L_{3-4}) of the two mosquito species.

The mean mortality in 5, 10 and 15mg was approximately similar to that of the control group. However, the mean mortality recorded for 20mg was substantially greater than all other concentrations. Hence, it was selected as the lower concentration in the subsequent tests.

2.7 Experiment:

The method described by Bukhari *et al*. (2010) was used. Briefly, the susceptibility of mosquito species, the effect of fungus species, larval stage and fungus concentrations were examined by recording the mortality of mosquito larvae caused by different concentration of *M. anisopliae* and *B.bassiana* conidia. This was done by comparing the mortality of early (L_{1-2}, 1-3 days old) and late (L_{3-4}, 4-8 days old) larval stages of *An. Arabiensis* and *Culex quinquefasciatus*. Fifty L_{1-2} or L_{3-4} larvae.

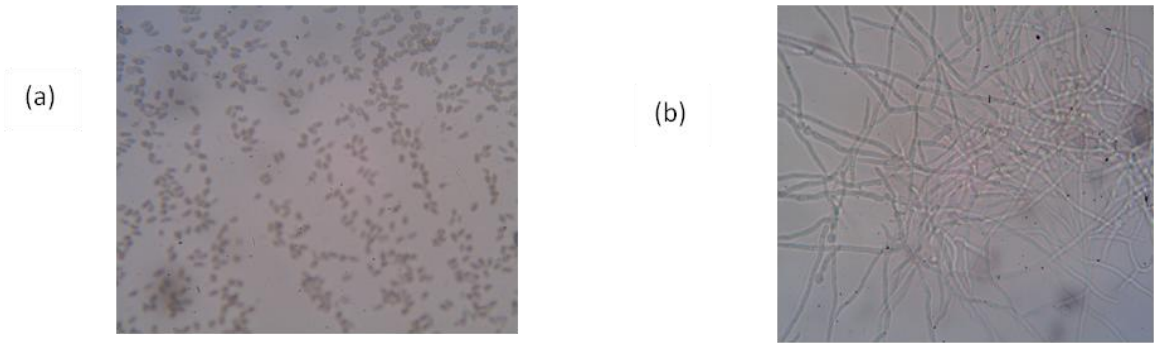

Figure 5: Germination of (a) *Metarhizium anisopliae* Var acridum and (b) *Beauveria bassiana* after 24 hours incubation at 27^{o}C$\pm$ 1^{o}C and relative humidity 65% $\pm$ 5%.

were placed into a hollow wire metal troughs 25 x 8 cm. Which was filled with one liter of dechlorinated tap water and were fed on tetramin (0.1- 0.2mg/larva/day) for L_{1-2} and (0.3 mg/ larva /day) for L_{3-4} (Benedict, 2007). On these troughs fungal conidia were dusted on water surface in different amounts i.e. 20 mg- 40 mg. Dead larvae and pupae were counted and removed daily for the next 10 days. The amount of food added was adjusted to the daily mortality and/or pupation. The resulting 20 treatments (two mosquito species x two developmental stages X four fungal concentrations and one control) were each replicated four times all replicates were carried out under the same conditions. Every replicate had a control and the four fungus concentrations. The same protocol was followed each time and the same batch of fungi was used for all these experiments. Some of the dead larvae were transferred to 70% alcohol as a first step for slide preparation. Following the procedure describe by Bancroft *et al*. (1984), 5 micron sections were made and then examined under 40X10 (Figure 6,7)

The collected data were statistically analyzed, then means were compared following the procedure described by Gomez and Gomez (1984). In addition, the 50% lethal period LT_{50} was calculated using the straight line equation

$$Y= a + b\ x$$

Where: Y= the period, a= intercept, b= slope of the curve, x= mortality percentage

3. **Results**

The mean mortality percent (Table 1 and 2) of *Anopheles arabiensis* (Sp_1) and *Culex quinquefasciatus* (Sp_2) subjected to 5 biological control treatments. Both mosquito species were susceptible to the two fungus species at both early and late larval stage.

An. arabiensis showed a high mortality percent compared to *C. quinquefasciatus*.

In case of fungus species (Table 1) the larvae which were exposed to *Metarhizium anisopliae*, had a higher mortality percent 76.27% and 83.9% for *An. arabiensis* and 48.16% and 56.11% for *C. quinquefasciatus* for the concentrations Ma_{20} and Ma_{40}, respectively, at day 10. In comparison to those exposed to Beauveria bassiana obtained 61.81% and 65.32% for An. arabiensis, and 38.02% , 45% for C.quinquefasciatus for the concentration Bb_{20} and Bb_{40}, respectively. However, this was apparent only in the late larval stage (L_{3-4}) of both *An. arabiensis* and *C. quinquefasciatus*.

The early larval stage (L_{1-2})showed that (Table 2) *An. arabiensis* and *C. quinquefasciatus* at early stage were more susceptible to the two fungus species than the late larval stage(L_{3-4}).

From Table 3, the estimated lethal period (LT_{50}) for the tested entomopathogenic fungi, indicated that the most virulent one was Ma_{40} having the least LT_{50} followed by Ma_{20}.

Table 1: Mean percent mortality of *An. arabiensis*(Sp_1) and *C. quinquefasciatus* (Sp_2) larvae (L_{3-4}) subjected to 5 biological control treatments, over a period of 10 days

Days	Species	Control	Ma_{20}	Ma_{40}	Bb_{20}	Bb_{40}	5%LSD
2	Sp1	1.81	3.39	20.77	1.81	3.39	SP 3.43
	Sp2	1.81	5.47	16.89	7.55	1.81	Treat 5.43
3	Sp1	3.39	13.47	33.04	7.05	14.49	Sp 5.50
	Sp2	1.81	18.88	24.73	8.83	3.39	Treat 8.70
4	Sp1	6.48	25.64	47.96	13.3	23.16	SP 7.05
	Sp2	3.39	23.45	27.34	9.21	19	Treat 11.15
5	Sp1	7.34	38.1	60.45	18.13	36.31	SP 6.91
	Sp2	6.55	22.88	31.7	15.14	27.14	Treat 10.93
6	Sp1	8.56	47.44	68	30.68	42.45	SP 5.64
	Sp2	7.4	33.48	37.65	24.16	31.34	Treat 8.92
7	Sp1	10.99	57.28	75.55	43.85	50.07	SP 4.31
	Sp2	11.06	37.72	45.03	33.43	37.28	Treat 6.82
8	Sp1	10.99	64.05	77.16	51.2	55.03	SP 4.81
	Sp2	13.42	41.82	50.27	36.23	40.92	Treat 6.61
9	Sp1	14.08	70.5	83.9	58.89	58.87	SP 3.21
	Sp2	15.21	45.57	53.82	37.73	43.55	Treat 5.07
10	Sp1	18.54	76.27	83.9	61.81	65.32	SP 4.02
	Sp2	15.21	48.16	56.11	38.02	45	Treat 6.36

Ma=Metarhizium anisopliae Bb=Beauveria bassiana Sp= Species Treat = Treatment

Table 2: Mean percent mortality of *An.arabiensis* (Sp_1) and *C.quinquefasciatus* (Sp_2) larvae (L_{1-2}) subjected to 5 biological control treatments, over a period of 10 days

Days	Species	Control	Ma_{20}	Ma_{40}	Bb_{20}	Bb_{40}	5% LSD
2	Sp_1	1.81	3.39	20.77	1.81	3.39	SP 3.43
	Sp_2	1.81	5.47	16.89	7.55	1.81	Treat 5.43
3	Sp_1	3.39	13.42	33.04	7.05	14.49	SP 5.50
	Sp_2	1.81	18.88	24.73	8.83	3.39	Treat 8.70
4	Sp_1	7.4	87.97	90	45.52	40.07	SP 3.21
	Sp_2	5.82	90	90	56.25	75.71	Treat 5.07
5	Sp_1	7.4	87.97	90	57.8	73.48	SP 3.21
	Sp_2	12.76	90	90	71.61	86.46	Treat 5.07
6	Sp_1	7.4	87.97	90	57.8	73.48	SP 3.21
	Sp_2	12.76	90	90	71.61	90	Treat 5.07

Days	Species	Control	Ma_{20}	Ma_{40}	Bb_{20}	Bb_{40}	5% LSD
7	Sp_1	7.4	87.97	90	63.31	73.48	SP 3.21
	Sp_2	12.76	90	90	71.61	90	Treat 5.07
8	Sp_1	7.4	90	90	90	90	SP 3.21
	Sp_2	12.76	90	90	90	90	Treat 5.07

Ma=*Metarhizium anisopliae* Bb=*Beauveria bassiana* Sp= Species Treat = Treatment

Table 3: Estimated lethal period (LT50) for An. arabiensis (Sp1) and C. quinquefasciatus (Sp2) subjected to 4 treatments.

Treatment	Sp1	Sp2
Ma20	7.47	7.75
Ma40	4.59	6.06
Bb20	8.19	9.47
Bb40	7.47	9.19

Ma=*Metarhizium anisopliae* Bb=*Beauveria bassiana*

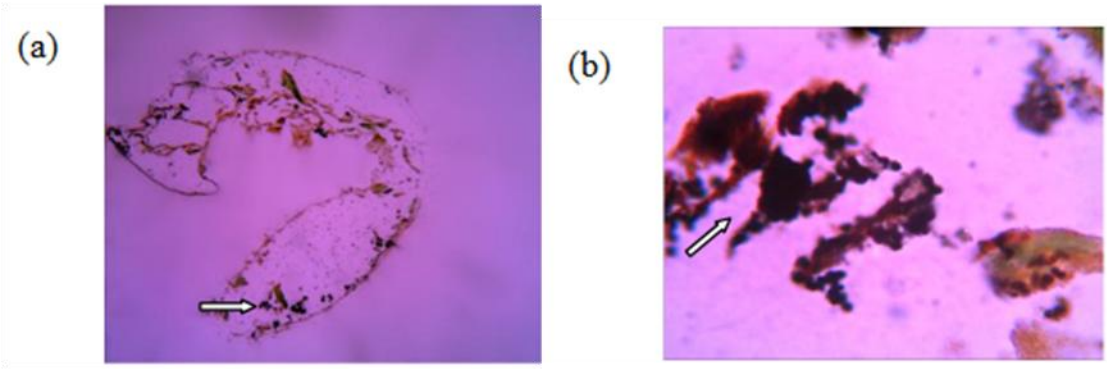

Figure 6: L.S of *An.arabiensis* larva showing spores of *Beauveria bassiana* (a) X10 (b) X40

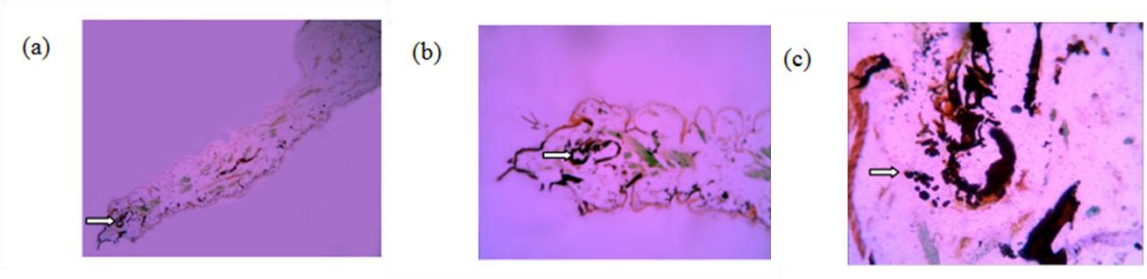

Figure 7: L.S of *An.arabiensis* larva showing presence of hyphal bodies and spores of *Metarhizium anisopliae* head region (a) X10, (b) X25, (c) X40.

4. **Discussion:**

The reason for the differences in mortality caused by *Metarhizium anisopliae* and *Beauveria bassiana* at the early larval stages (L_{1-2}) and the reduced susceptibility for the late larval stage (L_{3-4}) stage can be due to the fact that younger stages have a longer developmental time a head until pupation, during which they feed and molts two to three times. Larvae are more vulnerable to infection immediately after ecdysis, because of the soft cuticle. Younger larval stages thus have an increase probability of acquiring an infection. Late larval stages, on the other hand, have reduced food intake and thicker cuticle and thus they are less likely that spores could penetrate their body (Apperson *et al*., 1992; Lord and Fukuda, 1990). Also younger instars larvae possess more hydrophobic cuticle than the mature ones (Boucias *et al*., 1988). Furthermore, Boucias *et al*. (1988) found that when incubated the conidial suspension (10^7 conidia/ml) of the two fungi with first instars they completely coated with a monolayer of conidia, whereas substantially fewer conidia attached to the fourth-instar larval cuticle. Also conidia of these fungi (*M. anisopliae* and *B. bassiana*) possess a hydrophobic rodlet layer which may provide protection against dehydration, microbial attack and a means of dispersal in air current. The major role of the rodlet layer present on these fungi is the attachment of conidia to insect cuticle a process which is mediated by a hydrophobic interaction (Boucias *et al*., 1988). This finding is not unexpected, as in most insect-pathogen combination, insect decrease in susceptibility to infection by an equal amount of pathogens as they advance in development (Apperson *et al*., 1992). Though normally less susceptible to infection than early instars, the later instars are nevertheless, still susceptible, particularly just after molting prior to completion of sclerotization of the

cuticle (Apperson *et al*., 1992). Bukhari (2010), on his finding explain that, *Metarhizium anisophiae* var anisophae and *Beauveria bassiana* spores act as midgut toxins and enter the body mainly through the mouth when applied over the water surface they are readily available to the anopheline larvae, which are surface feeders. Another explanation is that conidia are hydrophobic, thus floating on water surface, and contact mosquito larvae that feed below the surface when they break the water tension with their perispiracular valves for air intake. The fungus germinates and penetrates into respiracal siphon, blocking the breathing mechanism (Lacey *et al*., 1988). These two reasons make anopheline larvae more susceptible than Culex larvae which support finding in this research. Furthermore Crisan (1971) and Roberts (1974) in their works found that dry conidia can be ingested. Also Miranpuri and khachatourians (1991) indicated that the head maybe an equally important infection site. When spores enter the larval body through the month or siphon they mechanically block these passages while the other attacking spores germinate releasing endotoxins as well as damaging the larval tissue with their vegetative growth (Hegedus *et al*., 1995). In this case there were a whole spectrum of offence that has to be tackled by the larval immune system. (The more variable the modes of action, the lower is the probability that resistance will develop against control agent (Mulla *et al*., 2003).

The differences in the rapidity of vegetative growth found in *Metarhizium anisopliae* and *Beauveria bassiana* may cause the difference in the observed effect of the two fungi. *Beauveria bassiana* isolates could be categorized into a group with high total sporulation and slow vegetative growth. While *Metarhizium anisopliae* isolates fell into another group with high quick vegetable growth although at the expense of sporulation (Sun *et al*., 2002). Slow vegetative growth may be associated with slow release of endotoxins inside the larval body and thus a delayed mortality. In laboratory tests (Alves *et al*., 2002) proved that *Beauveria bassiana* was ineffective against *Culex quinquefasciatus*. Also laboratory experiment reported by Ramoska (1982), shows that *Metarhizium anisopliae* suppressed Culex quinquefasciatus larval population for nearly a month. On the other hand, the same strain used by (Alves *et al*., 2002) had lost its effect on the same mosquito species after 3 days. These two reports also explain the finding in this experiment, that *Culex quinquefasciatus* was less susceptible to the two fungi.

Although there was a difference in the effect of fungal concentration this was not proportional. The fungal spores are hydrophobic and when applied over the water surface without a surfactant they clump together into masses that become dense over time. Larvae may have reject the spore mass as food most likely because of large clump size and the density of the mass made spore attachment avoidable. As a result, high concentrations, did not provide a better spatial and temporal coverage that could have resulted in increased mortality rate (Bukhari *et al*., 2010).

The findings described in this research are encouraging and may form the basis for further research on this topics such as: The search for more virulent endogenous fungi, in water where mosquito breeds or in dead mosquito collected from the breeding site. Although *M. anisopliae* used in this research was found highly virulent against *An. arabiensis* , it is possible that other isolates of this species, or another Hyphomycetous species altogether will prove to be even more virulent. Studies on more efficient strain or species under the relevant field conditions. Improving conidial formulation may increase both conidial attachment to the insect cuticle, as well as increasing conidial longevity. Also the selection of appropriate method for application is very crucial to the success of the biocontrol agent.

This study showed that both fungus species have potential to kill mosquitoes (*Anopheles arabiensis* and *Culex quinquefasciatus*) in the larval stage, and mortality rate depends on fungus species itself and larval stage targeted. However, increasing the concentration of fungal spores did not show a proportional increase in mortality rate. High fungus concentration result in clumped spores which did not provide uniform coverage over water surface.

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