Mosquito larvicidal, pupicidal and field evaluation of microbial insecticide, *Verticillium lecanii* against the malarial vector, *Anopheles stephensi*

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ABSTRACT

Anopheles stephensi is the primary vector of malaria in India and other West Asian countries and improved methods of control are urgently needed. The present study clearly explored that the larvicidal, pupicidal and field evaluation effects of entomopathogenic fungi *Verticillium lecanii* against the malarial vector, *A. stephensi*. Lethal dose concentrations (LC_{50} and LC_{90}) were calculated for different larval instars and pupal stages. The LC_{50} and LC_{90} values were represented as follows: LC_{50} value of first instar larvae was 105.65%, second instar larvae was 131.58%, third instar larvae was 170.19%, fourth instar larvae was 193.44% and pupa was 229.52% respectively. LC_{90} value of first instar larvae was 307.60%, second instar larvae was 349.72%, third instar larvae was 400.30%, fourth instar was 415.18% and pupa was 458.57%, respectively. *V. lecanii* showed the highest mortality rate against the mosquito larvae in laboratory and field. The larval density was decreased after the treatment at the breeding sites, and hence, this fungal insecticide is the suitable alternatives of synthetic insecticides for the mosquito vector management.

Keywords: Entomopathogenic fungi, *Verticillium lecanii*, *Anopheles stephensi*, larvicide, field efficacy

INTRODUCTION

Mosquitoes are responsible for the transmission of more diseases than any other group of arthropods and play an important role as etiologic agents of malaria, filariasis, dengue, yellow fever, Japanese encephalitis and other viral diseases [1]. Mosquito-borne diseases are endemic in more than over 100 countries, causing mortality of nearly two million people every year and at least one million children die of such diseases each year, leaving as many as 2100 million people at risk around the world. Mosquitoes alone transmit disease to more than 700 million people annually [2].

Malaria is the largest single component of disease burden; epidemic malaria, in particular, remains a major public health concern in tropical countries. In many developing countries and especially in Africa, malaria exacts an enormous toll in lives, in medical costs and in days of labor lost [3]. Malaria now is responsible for the estimated more than 300 million people falling ill, and there are one million deaths per year [4]. *A. stephensi* is recognized as a major vector for urban malaria in India. This species prefers to breed in small synthetic water collections and is responsible for frequent outbreaks of malaria, particularly at construction sites in urban areas [5]. Mosquito transmitted disease continues to be a major source of illness and death. Most parasitic diseases are

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tropical and the intensifying globalization and climatic change are increasing the risk of contracting arthropod-borne illnesses [6]. Malaria is a leading cause of morbidity and mortality in Ethiopia. Besides, it is one of the major obstacles to socioeconomic development as the main transmission periods coincide with peak agricultural and harvesting period [7]. There is a critical need for cheap and effective control campaigns, as were implemented during the DDT era. A creative and organized search for new strategies, perhaps based on new technologies, is urgently required irrespective of future climate change [8].

Mosquito control, in view of their medical importance, assumes global importance. In the context of ever increasing trend to use more powerful synthetic insecticides to achieve immediate results in the control of mosquitoes, an alarming increase of physiological resistance in the vectors, its increased toxicity to non-target organism and high costs are noteworthy [9]. Most of synthetic chemicals are expensive and destructive to the environment and also toxic to humans, animals and other non-target organisms. Besides, they are nonselective and harmful to other beneficial organisms. Some of the insecticides act as carcinogenic agents and are even carried through food chain which in turn affects the non-target organism. Therefore alternative vector control strategies, especially effective and low cost are extremely imperative [10,11].

Biopesticides provide an alternative to synthetic pesticides because of their generally low environmental pollution, low toxicity to humans, and other advantages [12]. Entomopathogenic fungi comprise approximately 700 species of Zygomycetes, Ascomycetes and Deuteromycetes [13]. Within the group of Deuteromycetes, fungi belonging to the Hyphomycetes have simple, non-sexual life cycles and are typically opportunistic pathogens with a broad insect host range [14]. The impact of persistence of entomopathogenic fungi on insects and on filage has not been extensively studied. Conidia of hyphomycetous fungi strongly adhere to insect cuticle, and the attachment of conidia to cuticles is through to involve nonspecific adhesion mechanisms mediated by the hydrophobicity of the cell wall [15]. In general, the life cycle of the entomopathogenic fungi involves an infective spore stage, which germinates on the cuticle of the host, forming a germ tube that penetrate the cuticle and invades the hemocoel of the insect host [13].

The microorganisms are kept under consideration and these are used as biocontrol agents against insects and special emphasis is put on *Verticillium lecanii* because of its qualities like shelf life, mode of action, non toxic effects to environment, high host specificity and non persistence. It forms mycelium which contains spores and these spores are called conidia and are used for characterization. Factors like germination rate, growth, speculation are the indicators of virulence [16]. *Verticillium lecanii* is an entomopathogenic fungus. The mycelium of this fungus as a active ingredient of cyclodepsipeptide toxin called bassianolide and other insecticidal toxins such as dipcolonic acid, hydroxycarboxylic acid, cyclosporine which infect of mosquitoes, rust fungi, scale insects and lead to death the host. The spores of this fungus when come in contact with the cuticle (skin) of target insects, they germinate and grow directly through the cuticle to the inner body of their host. The fungus proliferates throughout the insect's body, draining the insect of nutrients, and eventually killing it in around and a week time. Thus, there is a need to develop biopesticides that are effective, biodegradable and do not leave any harmful effect on the environment.

More recently, studies of fungal infection in adult mosquitoes (mostly malaria vectors) have demonstrated potential for fungi to reduce longevity [17-19], blood-feeding success, fecundity [20] and vector competence [21]. Other recent empirical studies demonstrate that fungal pathogens can infect insecticide resistant mosquitoes and reduce expression of insecticide resistance [22] and act synergistically with conventional interventions such as bednets [23,24] and chemical insecticides [22,25], highlighting the potential for using entomopathogenic fungi in novel integrated strategies for adult vector control.

Two isolates of *Verticillium lecanii* have been used successfully to control aphids on chrysanthemum and whitefly on cucumber and tomato [26]. Different products based on fungi

against a variety of insects have been commercialized in different countries of world: e.g. Mycotal and Vertalec contain different strains of *V. lecanii* and are being used against homopteran in glasshouses. Using entomopathogenic fungi as mycoinsecticides against aphids has been successful [27,28]. *Verticillium lecanii* (Zimm.) Viegas is a major microbial biocontrol agent of whiteflies and aphids. The purpose of the present investigation was to explore the mosquito control agent under laboratory as well as field conditions. The *Verticillium lecanii* is reported to have mosquitocidal properties of the control of the malarial vector, *A. stephensi*.

MATERIALS AND METHODS

Collection of eggs and maintenance of larvae

The eggs of *A. stephensi,* were collected from National Centre for Disease Control field station of Mettupalayam, Tamil Nadu, India, using an "O" type brush. These eggs were brought to the laboratory and transferred to 18 × 13 × 4 cm enamel trays containing 500-mL of water for hatching. The mosquito larvae were fed with pedigree dog biscuits and yeast at 3:1 ratio. The feeding was continued until the larvae transformed into the pupal stage.

Maintenance of pupae and adults

The pupae were collected from the culture trays and transferred to plastic containers (12 × 12 cm) containing 500 ml of water with the help of a dipper. The plastic jars were kept in a 90 × 90 × 90-cm mosquito cage for adult emergence. Mosquito larvae were maintained at 27±2°C, 75–85% RH under a photoperiod of 14 L: 10D. A 10% sugar solution was provided for a period of 3 days before blood feeding.

Blood feeding of adult mosquito *A. stephensi*

The adult female mosquitoes were allowed to feed on the blood of a rabbit (a rabbit per day, exposed on the dorsal side) for 2 days to ensure adequate blood feeding for 5 days. After blood feeding, enamel trays with water from the culture trays were placed in the cage as oviposition substrates.

Fungal bioassay

V. lecanii both liquid and solid formulation were obtained from Research and Development Centre, T. Stanes and Company Limited, Coimbatore. Liquid formulation contains 1×10^9 CFU/ml and Carrier formulation contains 1×10^8 CFU /gm of the materials. The required quantity of entmopathogenic fungi, *V. lecanii* liquid formulation was thoroughly mixed with distilled water to prepare at various conidia concentrations were adjusted 1×10^2 to 5×10^6 viable conidia/ml, respectively.

Larvicidal bioassay test of *Verticillium lecanii*

A laboratory colony of *Anopheles stephensi* larvae were used for the larvicidal activity. Twenty five numbers of first, second, third and fourth instars larvae were kept in 500 ml glass beaker containing 249 ml of dechlorinated water and 1ml of desired concentration of *V. lecanii* was added. Larval food was given for the test larvae. At each tested concentration 2 to 5 trails were made and each trial consisted of three replicates. In the *V. lecanii* toxicity the larvae exposed dechlorinated water

without acetone served as control. The control mortalities were corrected by using Abbott's formula [29]:

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae introduced}} \times 100$$

The values of LC_{50}, LC_{90} and their 95% confidence limit of upper confidence limit (UCL) and lower confidence limit (LCL) , regression and chi-square values were calculated by using probit analysis [30] and the levels of significance by Duncan's Multiple Range Test.

Pupal Toxicity test

A laboratory colony of *Anopheles stephensi* pupae were used for pupicidal activity. Twenty five numbers of freshly emerged pupae were kept in 500 ml glass beaker containing 249 ml of dechlorinated water and 1 ml of *V. lecanii* was added. Five replicates were set up for each concentration and dechlorinated water without acetone served as control. The control mortality was corrected by Abbott's formula [29]:

$$\text{Corrected mortality} = \frac{\text{Observed mortality in treatment - Observed mortality in control}}{\text{100 - Control mortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{Number of dead pupae}}{\text{Number of pupae introduced}} \times 100$$

The values of LC_{50}, LC_{90} and their 95% confidence limit of upper confidence limit (UCL) and lower confidence limit (LCL), regression and chi-square values were calculated by using probit analysis [30] and the levels of significance by Duncan's Multiple Range Test.

Field trail

For the field trial the quantity of plant residues required (based on laboratory LC_{90} values) for each treatment was determined by calculating the total surface area of the water in each habitat. The required quantities of *V. lecanii* was mixed thoroughly with water in a bucket with constant agitation. Field applications of the *V. lecanii* was done with the help of a knapsack sprayer uniformly on the surface of the water in each habitat. Dipper sampling and counting of larvae monitored the larval density before 72 hrs, 96 hrs and 120 hrs after the treatment. A separate sample was taken to determine the species composition of each larval habitat. Twelve trails were conducted for *V. lecanii* alone treatment. The percentage of reduction was calculated by the following formula:

$$= \frac{C - T}{C} \times 100$$

where, C - is the total number of Mosquitoes in control, T - is the total number of mosquitoes in treatment.

Statistical analysis

All data were subjected to analysis of variance; the means were separated using Duncan's multiple range tests [31]. The average larval and pupal mortality data were subjected to probit analysis for calculating LC_{50}, LC_{90}, and other statistics at 95% fiducidal limits of upper fiducidal limit and lower fiducidal limit, and Chi-square values were calculated using the SPSS Statistical software package 13.0 version was used. Results with $P< 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Larvicidal and pupicidal activity of *Verticillium lecanii* (Zimm) Viegas, at various concentrations against *Anopheles stephensi* is shown in table 1. The considerable mortality was evident after the treatment of *V. lecanii* for all larval instars and pupae. Mortality was increased as concentration increased, for example, 29% mortality was noted at I^{st} instar larvae by the treatment of *V. lecanii* at 1×10^2 conidia/milliliter/liter, whereas it has been increased at 91% at 5×10^6 conidia/milliliter/liter of *V. lecanii* treatment. Similar trend has been noted for all the larval instars and pupae of *Anopheles stephensi* at different concentration of *V. lecanii* treatment. The LC_{50} and LC_{90} values were represented as follows: LC_{50} value of I^{st} instar was 105.65%, II^{nd} instar was 131.58%, III^{rd} instar was 170.19%, IV^{th} instar was 193.44% and pupa was 229.52% respectively. LC_{90} value of I^{st} instar 307.60%, II^{nd} instar was 349.72%, III^{rd} instar was 400.30%, IV^{th} instar was 415.18% and pupa was 458.57%, respectively. A total of 274 *A. stephensi* larvae found were observed in the drinking water systems. After being treated with *V. lecanii*, the *A. stephensi* larval density was reduced by 38.3%, 62.0%, 81.7% and 100% at 72, 96, 120 and 144 h, respectively; (Table 2). A total of 182 *A. stephensi* larvae found were observed in the drinking water systems. After being treated with *V. lecanii*, the *A. stephensi* larval density was reduced by 42.8%, 64.2%, 83.5% and 100% at 72, 96, 120 and 144 h, respectively (Table 3).

Viruses, bacteria and fungi can act as biocontrol agents against insects. The viral and the bacterial control agents infect insects via their digestive tract while fungi make entry into the host through the cuticle that is the outermost covering in insect and fungal cell wall. Insect-pathogenic fungi that act by contact and with no records of resistance developed so far could be a viable alternative. Louis Pasteur first recognized the potential of fungi for insect control. Over 400 species of fungi have been known to parasitize living insects. Most fungi that attack insects first make contact with the host in the form of conidia. Once the conidium attaches to the host, the fungus penetrates the insect cuticle with the help of hyphae produced from conidia. The fungal enzymes such as chitinases, proteases, lipases and others that weaken the cuticle accelerate the physical process of penetration. The cause of the insect's death is extensive fungal growth and production of different toxins in the haemolymph.

An extensive literature survey on important pests and pathogens of agriculture, importance of their control in terms of percent damage, importance of chemical and biological control strategies, disadvantages and limitations of chemical agents, the market status of the different chemical and biological control agents, current and alternative strategies to control plant pathogenic fungi and insects, eco-friendly technologies and importance of alternative biocontrol agents like fungi and cuticle/cell wall degrading enzymes along with the chitin metabolism inhibitors with special reference to the host-pathogen interaction, transsectorial issues, possibility of use of this eco-friendly package in organic farming and integrated pest management programs has been done. Wang et al. [32] studied virulence of six strains of *V. lecanii* against sweet potato whitefly *Bemisia tabaci*. Their results indicated that strains V16063, V3450 and Vp28 were virulent against *B. tabaci* having LC_{50} values of 2.57×10^5, 6.03×10^5 and 6.05×10^5 conidial/mL, respectively.

A number of entomopathogenic fungi have been so far used effectively to control mosquito vector for the last few decades. The efficacy of *Metarhizium anisopolie* ICIPE-30 and *Beauveria bassiana* I93-825 (IMI 391510) (2×10^{10}conidia/m^2) applied on mud panels (simulating walls of traditional Tanzanian houses), black cotton cloth and polyester netting was evaluated against adult *Anopheles gambiae* [33]. They concluded that both fungal isolates reduced mosquito survival on immediate exposure up to 28 d after application. The role of fungi *Beauveria bassiana* (Balsamo) metabolites for controlling malaria and filaria in tropical countries have been evaluated [34]. These metabolites were found to be more effective on *Anopheles stephensi (An. stephensi*) than *Cx. quinquefasciatus* larvae. Further, the pathogenicity of *Fusarium oxysporum* (*F. oxysporum*) against the larvae of *Cx. quinquefasciatus* (Say) and *An. stephensi* (Liston) in laboratory have been tested [35]. They could observe that the extracellular metabolites of *F. oxysporum* in Czapek Dox broth were the most effective against the first and fourth instars of *An. stephensi*. The third and fourth instars of *Cx. quinquefasciatus* were more sensitive than the first and second instars. The results of the present study showed that the extracellular metabolites of *F. oxysporum* were less effective against *An. stephensi* but highly effective against *Cx. quinquefasciatus* larvae. This may be due to the size of culex which increases more surface area.

Table 1. Larvicidal and pupicidal activity of *Verticillium lecanii* at various concentrations against malarial vector, *A. stephensi.*

Larval instars	% of Larval and pupal mortality ± SD Concentration of *V. lecanii* (conidia/milliliter/liter)					LC_{50} (LFL – UFL)	LC_{90} (LFL – UFL)	Regression equation	x^2
	1×10^2	2×10^3	3×10^4	4×10^5	5×10^6				
I	29±0.92[a]	41±0.52[a]	53±1.35[a]	69±1.97[a]	91±0.78[a]	105.65 (85.30-124.30)	307.60 (272.20-358.94)	X=0.006 Y=-0.670	1.252*
II	25±1.79[bc]	35±1.22[b]	48±1.05[b]	63±0.88[b]	85±0.65[b]	131.58 (111.18-151.84)	349.72 (307.91-411.61)	X=0.006 Y=-0.773	0.511*
III	21±0.69[c]	28±1.34[c]	37±1.08[c]	55±0.63[cd]	78±1.16[c]	170.19 (149.17-193.99)	400.30 (350.55-475.38)	X=0.006 Y=-0.948	0. 492 *
IV	16±0.47[d]	22±1.20[cd]	33±1.16[d]	52±0.74[e]	73±1.75[de]	193.44 (172.30-218.78)	415.18 (365.18-489.79)	X=0.006 Y=-1.118	0.027*
pupa	12±1.37[e]	17±1.02[e]	27±0.71[e]	44±0.94[f]	65±1.20[e]	229.52 (205.32-261.15)	458.57 (400.76-546.84)	X=0.006 Y=-1.284	0.019*

LCL – 95% Lower Confidence Limit, UCL – 95% Upper Confidence Limit; within the column means followed by the same letter(s) are not significantly different at 5% Level by DMRT.

Table 2. Field trail by using *Verticillium lecanii* against malarial vector, *A. stephensi*.

Larval Density				
Before Treatment	After treatment			
	3rd day	4th day	5th day	6th day
47	29	18	10	-
53	34	22	13	-
40	22	13	6	-
39	21	11	4	-
35	38	26	12	-
40	25	14	5	-
Total=274	169	104	50	-
Average=45.66	28.16	17.33	8.33	-
Reduction (-)	38.32%	62.04%	81.75%	100%

Place: Vadavalli, Coimbatore District, Tamil Nadu, India; Habitat: Over Head Water Tank; Size: 2 × 1.5m; Species: *Anopheles stephensi*; Stage: Larval; Calculation: 2 × 1.5 = 3.0 × 1.5 × 10 = 45.00; Required Concentration: 45.00 %.

Kamalakannan et al. [36] proved that the entomopathogenic fungus, *M. anisopliae* is being considered as a biocontrol agent for the adult mosquito of *A. stephensi* (malarial vector). In the present experiment was carried out in the laboratory of 30–50 male and female adult mosquitoes were exposed to *M. anisopliae* (exposed to 1×10^6 conidia/ml of oil or water suspension). Kovendan et al. [37] reported that 96% and 94% adult mortality was observed in oil and water formulated

conidia of *M. anisopliae*. In the present result, considerable mortality was evident after the treatment of *V. lecanii* for all larval instars and pupae. Mortality was increased as concentration increased, for example, 29% mortality was noted at Ist instar larvae by the treatment of *V. lecanii* at 1×10^{2} conidia/milliliter/liter, whereas it has been increased at 91% at 5×10^{6} conidia/milliliter/liter of *V. lecanii* treatment. Similar trend has been noted for all the larval instars and pupae of *Anopheles stephensi* at different concentration of *V. lecanii* treatment. The LC_{50} and LC_{90} values were represented as follows: LC_{50} value of Ist instar was 105.65%, IInd instar was 131.58%, IIIrd instar was 170.19%, IVth instar was 193.44% and pupa was 229.52% respectively. LC_{90} value of Ist instar 307.60%, IInd instar was 349.72%, IIIrd instar was 400.30%, IVth instar was 415.18% and pupa was 458.57% respectively. Earlier investigations were undertaken on ten microbial product to develop a strategy to control mosquito larval and pupal population in the lab and field [38]. Highest larval mortality was evident in the lab with LC_{50} and LC_{90} at 0.25 and 0.5 at 24 h for *A. aegypti*.

Table 3. Field trail by using *V. lecanii* against malarial vector, *A. stephensi.*

Larval Density				
Before Treatment	After treatment			
	3rd day	4th day	5th day	6th day
40	24	13	5	-
36	19	15	7	-
42	27	18	9	-
24	12	8	4	-
18	10	6	3	-
22	12	5	2	-
Total=182	104	65	30	-
Average=30.33	17.33	10.83	5	-
Reduction (-)	42.85%	64.28%	83.51%	100%

Place: Goundampalayam, Coimbatore District, Tamil Nadu, India; Habitat: Over Head Water Tank; Size: 2 × 1.5m; Species: *Anopheles stephensi*; Stage: Larval; Calculation: 2 × 1.5 = 3.0 × 2.0 × 10 = 60.00; Required Concentration: 60.00%.

Jonathan et al. [39] measured survival of *Ae. aegypti* infected with a high dose of FI-277 over different application methods. All substrates tested were capable of infecting mosquitoes. Most notably, spores spread manually onto black cotton had the greatest impact on mosquito survival, killing 100% of mosquitoes by day 8. We chose these substrates because they represent potential methods of application of infective spores to *Ae. aegypti* in the field. Black cloth has been used successfully in resting targets for *Ae. aegypt*i [40,19], so the efficacy of *B. bassiana* on black cloth suggests that the fungus has potential to control mosquitoes in the field using resting targets. Field applications of entomopathogenic fungi should take this into account; one potential solution is to use a higher dose to account for reduced exposure time, although studies by Mnyone et al. [41] found that increasing concentrations of *B. bassiana* isolate IMI391510 above 2×10^{10} spores m^{-2} and exposure time above 30 min did not appreciably increase mosquito mortality. In the present result reveals that, total of 182 *A. stephensi* larvae found were observed in the drinking water systems. After being treated with *V. lecanii*, the *A. stephensi* larval density was reduced by 42.8%, 64.2%, 83.5% and 100% at 72, 96, 120 and 144 h, respectively. A total of 274 *A. stephensi* larvae found were observed in the drinking water systems. After being treated with *V. lecanii*, the *A. stephensi* larval density was reduced by 38.3%, 62.0%, 81.7% and 100% at 72, 96, 120 and 144 h, respectively. In conclusion, entomopathogenic fungi *Verticillium lecanii* have displayed toxicity on different larval instars of *A. stephensi*. The study showed an increase in mortality with the increase in concentration, and the early instars larvae are much susceptible than the later ones. The results suggested that a smaller dose of entomopathogenic fungi *Verticillium lecanii* liquid formulation is sufficient and can be directly used in the dwelling habitats of mosquitoes for effective control.

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