Virulence of *Verticillium* sp. against mosquito vectors for malaria, filarial, and dengue

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**OBJECTIVE:** Entomopathogenic fungi like *Beauveria bassiana*, *Metarhizium anisopliae* have been significantly pathogenic for mosquito vectors. Although few have been used for control. Moreover, the genus *Verticillium* encompasses a cosmopolitan group of ascomycete fungi. It is a major plant pathogen and parasitic on other fungi and insects.

**METHODS:** The culture filtrates released from the *Verticillium lecanii* (MTCC 3692) were grown on potato carrot broth media. These filtrates were purified with Whatman-1 filter paper and flash chromatograph respectively.

**RESULTS:** The results demonstrated LC\(_{50}\), LC\(_{90}\), and LC\(_{99}\) values of 0.6, 4.2, and 4.86, for *Culex quinquefasciatus*, and 1.3, 2.32, and 3.36 (µL/cm\(^2\)) for *Anopheles stephensi* after exposure for 10 h. LT\(_{50}\) values were 6.76 for *Culex quinquefasciatus* and *Anopheles stephensi* 3.54 for *Anopheles stephensi*. Moreover, the *Aedes aegypti* were completely susceptible at all selected doses.

**CONCLUSIONS:** The fungal culture filtrates of *Verticillium* sp. can reduce malaria, dengue, and filarial transmission on a par with chemical insecticides providing efficient delivery system can be developed.

1. Introduction

A pressing need exists for additional tools in insect control, particularly as few new chemical pesticides are under development[1]. Moreover, rapidly emerging insecticide resistance is creating an urgent need for new active ingredients to control the adult mosquitoes that transmit malaria[2]. In this regard the fungal spores of entomopathogenic fungi have shown considerable promise by causing substantial mortality within 7-14 days of exposure. These fungal conidia have been significantly reduced disease vectors species of mosquitoes[3]. The critics have argued that 'slow acting' fungal biopesticides are incapable of delivering mosquito control in different parts of the world. Moreover, the potential of entomopathogenic fungi *Metarhizium anisopliae* for the control of adult *Aedes aegypti* (*Ae. aegypti*) has confirmed under field conditions[3].

Eight fungal entomopathogens have screened for the ability to kill anopheline mosquitoes. The fungi were applied by spraying containers of mosquitoes with an oil formulation of infectious spores. Upon contact with a mosquito, the fungal spores (conidia) start to develop and invade the mosquito, after which the fungus multiplies and kills its host within two weeks. Moreover, fungus infected mosquitoes were less likely to take subsequent blood meals than uninfected mosquitoes. Moreover, Blanford et al. and Scholte et al.[4,5] studies have been provided exciting new data that it is concerned the development of a new weapon in the war against malaria. These results were encouraging for further research on mosquito control. Entomopathogenic fungi shown promise as effective agents against adult mosquitoes. In addition, transgenic fungi have expressed anti-plasmodium effector molecule that can target the parasite inside its vectors[6]. Recently, *Metarhizium* has been engineered to act against malaria to directly kill the disease agent with in mosquito vectors and also effectively block onwards transmission[7].

The genus *Verticillium* encompasses a cosmopolitan group of ascomycete fungi. It is normally considered to be nonpathogenic in humans. *Verticillium* produces an antifungal compound vergosin and an antitumor antibiotic, as well as a wide variety of additional compounds used by various industries. The health effects resulting from fungal exposure may be dependent on dosage and cause of
exposure and vary on individuals. The growth of the pathogenic fungi can cause opportunistic diseases. They thrive more under humid, nutrient available areas and with favourable temperatures. They have strong anti-fungal activity. The antifungal metabolites of Verticillium sp. inhibited biomass growth of itself and pathogens in liquid culture. The strong antifungal activities against the phytopathogenic fungi Verticillium sp. have been protected to the host by producing secondary metabolites[8].

Malaria is transmitted to humans by the bite of infected female mosquitoes of more than 30 Anopheline species. An estimated 3.4 billion people are risk of malaria and 1.2 billion are at high risk. In high risk areas, more than one malaria case occurs per 1,000 population[9]. Approximately 3.5 billion people live in dengue endemic countries which are located in the tropical and subtropical regions of the world[10]. Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. The infection occurs when filarial parasites are transmitted to humans through Culex quinquefasciatus (Cx. quinquefasciatus). More than 1.3 billion people in eighty one countries worldwide are threatened by lymphatic filariasis[11]. Interesting the present study evaluated Verticillium sp. as new adulticide for controlling of malaria, filaria and dengue vectors.

2. Materials and methods

2.1. Collection and culture of Verticillium sp. 3692

The strain of Verticillium sp. was obtained from Microbial Type Culture Collection and Gene Bank (MTCC-3692) Institute of Microbial Technology, Chandigarh India. Verticillium sp. were maintained on autoclaved Potato Carrot Broth (PCB) media (potato scrubbed and diced 20.0 g, carrot peeled and grated 20.0 g, deionized water 1000 mL and adjust pH to 7.2 with KOH). The broth was supplemented with 50 µg/mL chloramphenicol as a bacteriostatic agent. The colonies of Verticillium sp. were grown on PCA solid medium plates were transferred to each flask using an inoculation needle. The conical flasks, inoculated with Verticillium sp. were incubated at 25 °C for 15 days (Figure 1).

Figure 1. The culture of Verticillium sp. 3692 in PCB maintained in the laboratory.

2.2. Filtration of culture filtrates

The culture filtrates were obtained by filtering the broth through Whatman No. 1 filter paper. These metabolites were further filtered with the flash chromatograph. In the Flash chromatograph, a plastic column were filled with silica gel, with the sample to be separated placed on top of this support. The rest of the column was filled with an isocratic or gradient solvent which, with the help of pressure, enabled the sample to run through the column and became separated. Flash chromatography used air pressure initially, to speed up the separation.

2.3. Bioassays

The bioassays were carried out with laboratory reared Cx. quinquefasciatus, Ae. aegypti, and Anopheles stephensi (An. stephensi) as per the standard procedures recommended by World Health Organization with some respected modifications[12]. The freshly emerged three days old sugar fed adults were used for the assay. The five different volumes of 1.6, 2.2, 2.7, 3.2, and 3.8 µL/cm² of metabolites were sprayed in a cage (25 cm length × 15 cm width × 5 cm depth) containing 25 mosquitoes. The exposed mosquitoes were kept under observation, and dead mosquitoes were discarded daily. Each bioassay including control was conducted in triplicate on different days. In the control cages deionized water was sprayed. Daily mortality counts were performed. The bioassays were carried out at room temperature with 75% ± 5% relative humidity. The negative control was deionized water with 1% PCB while the positive control was Gokilhaht®-S 5EC (d,d-trans-cyphenothrin).

Figure 2. The infected Cx. quinquefasciatus, Ae. aegypti and An. stephensi with the culture filtrates of Verticillium sp. under the cage.

2.4. Statistical analysis

The efficacy study of the filtrate metabolites of Verticillium sp. were assessed against Cx. quinquefasciatus, Ae. aegypti, and An. stephensi by probit analysis with the statistical package IBM SPSS 19.0[13].

3. Results

In the present investigation we had evaluated the lethal effect of culture filtrates of Verticillium sp. against Cx. quinquefasciatus and Ae. aegypti after exposure of 12 h. The Whatman No.1 filtrates were recorded LC₃₀, LC₅₀, and LC₉₀ 0.71, 4.2, and 5.2 µL/cm² with the mortality rate (R²) 0.925. When these culture filtrates were again filtrated with the flash chromatograph the LC₃₀, LC₅₀, and LC₉₀, 0.6, 4.2, and 4.8 µL/cm² were recorded with the mortality rate 0.754. Moreover, the LT₃₀ and LT₉₀ 1.94, and 6.76 h at 4.7 mL. Similarly, these filtrates were used against An. stephensi the LC₃₀, LC₅₀, and LC₉₀ values 1.3, 2.7, and 3.36 µL/cm² respectively with the mortality rate (R²) 0.851. Moreover, the flash chromatograph filtered LC₃₀, LC₅₀, and LC₉₀ were 1.3, 2.32, and 3.36 µL/cm² with the R² value 0.904. Whereas, Watamman-1 filtrates have shown the
100% mortalities against *Ae. aegypti* after exposure of 7 h (Table 1). Moreover, infected *Cx. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* with the culture filtrates of *Verticillium* sp. were observed under the cage (Figure 2). The significant percent mortality were observed at tested concentrations after exposure of 7 h (Figures 3 and 4).

Table 1

| Mosquito               | LC₅₀ (µL/cm²) | LC₅₀(µL/cm²) | LC₅₀(µL/cm²) | LC₅₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) | LC₉₀(µL/cm²) |
|------------------------|---------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| *Cx. quinquefasciatus* | 0.74          | 4.20         | 5.20         | 3.98         | 8.91         | 0.60         | 4.20         | 4.86         | 1.94         | 6.76         |
| *An. stephensi*        | (0.11-1.36)   | (3.66-4.86)  | (4.60-5.80)  | (2.91-5.05)  | (6.92-10.9)  | (0.030-1.28) | (3.66-4.86)  | (4.03-5.24)  | (0.80-3.82)  | (5.62-7.90)  |
| *Ae. aegypti*          | 1.30          | 2.70         | 3.36         | 1.30         | 3.22         | 1.30         | 3.36         | 1.58         | 3.54         | 1.00         |
| (0.81-1.90)            | (2.12-2.32)   | (2.80-3.91)  | (0.816-1.92) | (1.77-2.88)  | (2.80-3.90)  | (0.41-2.75)  | (2.37-4.41)  |

*100% mortality.

Figure 3. Mortality of (*Verticillium* sp.) against *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* plotted as function of time post exposure.

Figure 4. Effects of culture filtrates of *Verticillium* sp. against *Cx. quinquefasciatus*, *An. stephensi*, and *Ae. aegypti* at different doses.

4. Discussion

The laboratories and field studies have been successfully demonstrated that entomopathogenic fungi can efficiently kill vectors of malaria, dengue, and filaria[14-18,1]. The biopesticides have been used of comprising entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi may decrease disease transmission by reducing mosquito vector longevity and also occur worldwide[19]. Many isolates have not been tested for virulence against mosquitoes. In this concern in our Laboratories, seventy eight isolates of entomopathogenic fungi representing twenty species were screened as potential biological control agents of *Ae. aegypti*, *An. stephensi*, and *Cx. quinquefasciatus* larvae and adults. Now we have one more species of *Verticillium* sp. has found considerable virulence against adults of *Ae. aegypti*, *Cx. quinquefasciatus*, and *An. stephensi* in the laboratories. Most of these fungal strains have shown lethal effect after exposure of 24, 42 and 72 h. The culture filtrates *Aspergillus niger* has been found pathogenic in 7.0 h against *An. stephensi*, *Ae. aegypti*, and *Cx. quinquefasciatus*(17). In the present investigations, the mortality rates were 100% against *Ae. aegypti*, while 95% against *Cx. quinquefasciatus* and *An. stephensi* after exposure of 7 h. Based on these efficacy data this fungal strain can be selected, for the control of adult mosquitoes with observed doses.

*Beauveria bassiana* has been expressed trypsin modulating oostatic factor exhibited increased virulence against *Anopheles gambiae* and *Ae. aegypti* related to the wild type strain. Utility of entomopathogenic fungi has expressed mosquito specific molecules to improve their biological control activities against mosquito vectors of diseases(20). The pathogenic fungi produce a wide variety of toxic metabolites, which vary from low molecular weight products of secondary metabolism to complex cyclic peptides and proteolytic enzymes(21). The fungal metabolites can be more effective by joint action of numerous toxins and enzymes(17). Another point to consider regarding bioassays is that an exposure time of 24 h, which is adequate for standard screening, may not be achievable in the field. While it may be possible for mosquitoes in resting boxes, such as those designed for using against exophilic *Anopheles arabiensis* in Tanzania, to stay exposed to fungus-treated surfaces for several hours or more, mosquitoes landing on a visual target may only rest on spores for as little as a few minutes(22). Entomopathogenic fungus *Leptolegnia chapmanii* has shown significant reduction in the population of *Ae. aegypti*(23). Recently, virulence for engineered *Metarhizium* or similar pathogens, and that all available information regarding the population ecology of the combined mosquito fungus system has been considered(7). Field applications of entomopathogenic fungi should take it into account, one potential solution is to use a higher dose to account for reduced exposure time. In *Aspergillus niger* the “ochratoxin” mycotoxin can be fast acting metabolites for controlling of adult mosquitoes(17). The nonchemical approaches to the management of malaria, dengue, and filaria are limited by site specificity of available methods. Similarly, fungi biopesticides have significant potential to achieve reduction in transmission comparable with those achieved with existing instant kill insecticides(24). An understanding of the modes of action of these methods will likely improve their range and efficacy. And efforts should continue to fully explore the operational feasibility.
of this alternative approach for mosquito control. The myco-
control technology is encouraging for extensive future research. Further investigations need to be excreted to translate laboratory promising results of many of the microbial agents into field control agents[25]. An important area of future research also involves examining *Verticillium* sp. for the determinants of race specificity. Similarly all these novel findings could be implemented with a time application with its fast acting impact against *An. stephensi*, *Cx. quinquefasciatus*, and *Ae. aegypti* population.

**Conflict of interest statement**

We declare that we have no conflict of interest.

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Several fungi have recently been used for adult mosquito control. The authors attempt to use new taxa of fungus for this purpose.

**References**

[1] Fan Y, Borovsky D, Hawkings C, Ortiz-Urquiza A, Kehyani NO. Exploiting host molecules to augment mycoinsecticide virulence. *Nat Biotechnol* 2012; 30: 35-7.

[2] Blanford S, Shi W, Christian R, Marden JH, Koekemoer LL, Brooke BD, et al. Lethal and pre-lethal effects of a fungal biopesticide contribute to substantial and rapid control of malaria vectors. *PLoS ONE* 2011; 6: e23591. doi: 10.1371/journal.pone.0023591.

[3] Caroline AT, Paula AR, Silva CP, Batt TM, Samuels RI. Monitoring persistence of the entomopathogenic fungus *Metarhizium anisopliae* under simulated field conditions with the aim of controlling adult *Aedes aegypti* (Diptera: Culicidae). *Parasit Vectors* 2014; 7: 198.

[4] Blanford S, Chan BH, Jenkins N, Sim D, Turner RJ, Read AF, et al. Fungal pathogen reduces potential for malaria transmission. *Science* 2005; 308: 1638-41.

[5] Scholte EJ, Ng’habi K, Kihonda J, Takken W, Paujimans K, Abdulla S, et al. An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* 2005; 308: 1641-2.

[6] Abdul-Ghani R, Al-Mekalla M, Ablasi MS. Microbial control of malaria: biological warfare against the parasite and its vector. *Acta Trop* 2012; 121: 71-84.

[7] Konard BP, Lindstrom M, Gumpinger A, Zhu J, Coombs D. Assessing the optimal virulence of malaria-targeting mosquito pathogens: a mathematical study of engineered *Metarhizium anisopliae*. *Malar J* 2014; 13: 11.

[8] You F, Han T, Wu JZ, Huang BK, Qin LP. Antifungal secondary metabolites from endophytic *Verticillium* sp. *Biochimica et Biophysica Acta* 2009; 179: 162-5.

[9] World Health Organization. *World Malaria reports* 2013. Geneva: World Health Organization; 2013. [Online] Available from: http://www.who.int/malaria/publications/world_malaria_report_2013/en/ [Accessed on 12th January, 2015]

[10] World Health Organization. Guidelines on the quality, safety and efficacy of dengue tetravalent vaccine (Live attenuated). Geneva: World Health Organization; 2011. [Online] Available from: http://www.who.int/ihr/publications/BS1215_Denguewithline_numbers_TRS_v24-18Jul2011_sub2.pdf [Accessed on 12th January, 2015]

[11] World Health Organization. Lymphatic filariasis. Geneva: World Health Organization; 2011. [Online] Available from: http://www.who.int/mediacentre/factsheet/fs102/en [Accessed on 12th January, 2015]

[12] World Health Organization. Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets. Geneva: World Health Organization; 2006. [Online] Available from: http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_WHOPES-GCDPP_2006.3_eng.pdf [Accessed on 12th January, 2015]

[13] Finney DJ. *Probit analysis*. 3rd ed. London: Cambridge University Press; 1971.

[14] Kanzok SM, Jacobs-Lorena M. *Entomopathogenic fungi* as biocontrol insecticides to control malaria. *Tren Parasitol* 2006; 22: 49-51.

[15] Singh G, Prakash S. Efficacy of the Trichophyton aquellio and *Lagenidium giganteum* metabolites against mosquitoes after flash chromatography. *Parasitol Res* 2012; 110(5): 2053-60.

[16] Singh G, Prakash S. Studies on fungal cultural filtrates against adult *Culex quinquefasciatus* (Diptera: Culicidae) a vector of filariasis. *J Parasitol Res* 2011; doi: 10.1155/2011/147373.

[17] Singh G, Prakash S. Evaluation of culture filtrates of *Culicinomyces clavispors*: Mycoadulticide for *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi*. *Parasit Res* 2012; 110: 267-72.

[18] Singh G, Prakash S. Lethal effects of *Aspergillus niger* against mosquitoes vector of filaria, malaria, and dengue: a liquid mycoadulticide. *Sci World J* 2012; doi: 10.1100/2012/603984.

[19] Darbro JM, Graham RI, Kay BH, Ryan PA, Thomas MB. Evaluation of entomopathogenic fungi as potential biological control agent of the dengue mosquito, *Aedes aegypti* (Diptera: Culicidae). *Biocont Sci Technol* 2011; 21: 1027-47.

[20] Kamareddine L, Fan YH, Osta MA, Keyhani NO. Expression of trypsin modulating oosomatic factor (TMOF) in an entomopathogenic fungus increase its virulence toward *Anopheles gambiae* and reduces fecundity in the target mosquito. *Parasit Vectors* 2013; 6: 22.

[21] Demain AL, Fang A. The natural functions of secondary metabolites. In: Fiechter A, editor. *History of modern biotechnology I: advances in biochemical engineering and biotechnology*. Berlin: Springer Berlin Heidelberg; 2000. p. 1-39.

[22] Lwetoijera DW, Sumaye RD, Madumula EP, Kavirse DR, Mnyone LL, Russel TL, et al. An extra-domiciliary method of delivering entomopathogenic fungus, *Metarhizium anisopliae* IP 46 for controlling adult population of the malaria vector, *Anopheles arabiensis*. *Parasit Vectors* 2010; 3: 18.

[23] Pelizza SA, Scorzetti AC, Tranchida MC. The sublethal effects of the entomopathogenic fungus *Leptolegnia chapmanii* on some biological parameters of the dengue vector *Aedes aegypti*. *J Insect Sci* 2013; 13: 22.

[24] Lynch PA, Grimm U, Thomas MB, Read AF. Prospective malaria control using entomopathogenic fungi: comparative evaluation of impact of transmission and selection for resistance. *Malar J* 2012; 11: 383.

[25] Singh G, Prakash S. New prospective on fungal pathogens for mosquitoes and vector control technology. *J Mosq Res* 2014; 4: 36-52.