Comparative efficacy and pathogenicity of keratinophilic soil fungi against *Culex quinquefasciatus* larvae

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Abstract Out of seven fungal species belonging to four genera isolated from pond and wallow soils using feathers of *Pavo cristatus* as bait, four species viz., *Aspergillus flavus*, *Aspergillus niger*, *Chrysosporium pseudomerdarium* and *Trichophyton ajelloi* were most frequent. *Chrysosporium* and *Trichophyton* spp. were more pathogenic on *Culex quinquefasciatus* larvae than *Aspergillus* and *Penicillium*. The bioefficacy tests conducted as per the protocol of World Health Organization and the LC₅₀ values calculated by the Probit analysis showed that 3rd-instar *C. quinquefasciatus* were more susceptible to the conidia of above fungi. Highest mortality was observed in the larvae of *C. quinquefasciatus* when exposed to *T. ajelloi*. The density of fungal conidia was greatest on the ventral brush, palmate hair and anal region of the mosquito larvae after exposing for 72 hours. The potentiality of these fungi for use in the control of *C. quinquefasciatus* is discussed which can be exploited as a suitable biocontrol agent in the tropics.

Keywords *Culex quinquefasciatus* · Entomopathogenic fungi · Instars · Mosquitoes

Introduction

Human lymphatic filariasis is of public health importance in many tropical and subtropical countries [1–2]. In India alone, about 553 million people are believed to be at risk of infection with the nematodes that cause the disease and approximately 48 million are already infected [3]. *Culex quinquefasciatus* and *Anopheles stephensi* are the most important man-biting mosquitoes in India. The former acts as a filariasis vector whereas the later works as a vector for malaria. The use of entomopathogenic fungi as biological control of mosquito larvae has been extensively studied [4–6]. However it is useful to record more information about soil fungi as biological control agents. Large numbers of keratinophilic soil fungi have been isolated by many workers from Indian soils, however, their utility as biological control agent of mosquito still unknown [7–8]. So the present study was conducted to generate more information on the pathogenic behaviour of the endemic keratinophilic fungi against *C. quinquefasciatus*.

Material and methods

Isolation of fungi and separation of conidia

Ten soil samples were collected from nearby ponds and wallows in Agra, India, where mosquitoes breed. The samples were collected between August to September when the ambient temperature ranged between 20–35°C. The soil samples were dig 5 cm below the surface by a sterile scalpel and collected in clean sterile plastic bags. Fifty grams of soil sample from each source was placed in a separate petri

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plates with five replications in each case. The keratinophilic soil fungi were isolated by feather baiting technique [9]. Feathers of *Pavo cristatus* (Indian peacock) were used as bait. Two grams of typical feather from the tail region of *P. cristatus* were used as bait. The feathers were cut in to 5 cm long pieces by scissors, sterilized in an autoclave, placed on the soil surface and moistened (5 ml/petri plate) with sterile distilled water. Petri plates were placed at $24 \pm 2^\circ\text{C}$ in incubators. After fifteen days, fungal colonies growing on the on the feathers were purified by transferring individual colony to petri plates containing Sabouraud dextrose agar (Ranbaxy, SAS Nagar, India) under aseptic conditions. The Sabouraud dextrose agar was supplemented with 25 $\mu\text{g/ml}$ active ingredient of chloramphenicol as bacteriostatic agent [5]. The isolates were identified as per the standard descriptions of Van Oorshot [10] (*Chrysosporium* spp.), Raper and Fennell [11] (*Aspergillus* spp.), Frey et al. [12] (*Trichophyton* spp.) and Domsch et al. [13] (other fungi). The colonies of fungi grown on Sabouraud dextrose agar for fifteen days were used for the separation of conidia and mycelia [14–15]. The hydrophobic dry conidia were used in the efficacy test and were without any wetting agent. The wetting agents will submerge the conidia in the water and to avoid this we did not use any wetting agent in the present study.

Mosquito rearing

C. quinquefasciatus colonies were maintained in the laboratory at a temperature of $25 \pm 2^\circ\text{C}$, relative humidity of $70 \pm 5\%$ and photoperiod of 14:10 hour (Light : Dark). Larvae were placed in sterile distilled water at pH 7.0. To counteract evaporation, water was added daily. Larvae were fed 0.4ml/beaker of a 5% (w/v) autoclaved suspension of freeze-dried yeast in distilled water on day-1 and day-2.

Bioassays

In a preliminary efficacy test against instars of *C. quinquefasciatus*, the mortalities of all fungi were recorded separately at a concentration of 10^5 conidia/ml. The efficacies of fungi against *C. quinquefasciatus* at the above concentration impart the value of six concentrations to be used. After fifteen days of growth, all fungi were tested against 3rd-instar because 3rd-instar *C. quinquefasciatus* were more susceptible than the 1st-, 2nd- and 4th-instar against all the fungi. Therefore in the present investigation we have used the 3rd-instar larvae of *C. quinquefasciatus*. In all the bioassays, twenty 3rd-instar mosquitoes were added to 500 ml beaker. Larvae were exposed to six serial dilutions of fungal spores chosen to produce larval mortalities between 20 to 95% for calculating the LC_{50} values. Each of the fungi was tested separately against larvae of *C. quinquefasciatus*.

The experiments were repeated thrice on different days of a month. The control larvae were fed the same food given to the experimental larvae. The larvae were washed twice with sterile distilled water to avoid any chance of fungal infection. Every 12 hours, all dead larvae were counted and removed from the test medium. Following incubation, dead larvae were rinsed with sterile water to eliminate non attached conidia. Thereafter dead larvae were kept in the cotton blue stain for 5 minutes. Stained dead larvae were examined individually under a microscope for fungal infection. The mortalities were recorded after three days based on the number of live larvae remaining in each beaker.

Data analysis

Control mortalities were subjected to Abbott's formula. Dosage mortality responses were subjected to the probit analysis method of Finney [16] and the LC_{50} with 95% fiducial limits, slope and regression lines were computed with the help of Microsoft Excel and SPSS.

Results and discussion

Seven fungal species in four genera were isolated from the samples of soil by the feather baiting technique using feathers of *P. cristatus* as bait (Fig. 1). *Aspergillus* was the most frequently isolated genus and comprised 43% of total isolated species. Among the three species, *Aspergillus niger* was the most frequently isolated species, the other two were

Fig. 1 Front view of soil fungal colonies after a growth of fifteen days on feather (*Pavo cristatus*) bait.

Aspergillus flavus and *Aspergillus fumigatus*. Of the two *Chrysosporium* species, *Chrysosporium pseudomerderium* van Oorschot was most frequently recorded species. The only *Penicillium* and *Trichophyton* species isolated were *Penicillium chrysogenum* and *Trichophyton ajelloi*. Saxena et al. [8] isolated 33 species of fungi classified in to 11 genera from Indian soil including the four genera which were isolated in the present study. Deshmukh and Verekar [7] isolated seven fungal species and out of which four are *Chrysosporium* spp. Most of the studies on keratinophilic fungi are generally reporting in nature, however their usefulness is reported in this paper.

Comparison of LCs of seven fungal species showed that larvae of *C. quinquefasciatus* were highly susceptible to *T. ajelloi* (Table 1). Among the *Aspergillus* spp. highest mortality of *C. quinquefasciatus* larvae was recorded against *A. flavus* and lowest in *A. fumigatus*. *Chrysosporium* and *Trichophyton* spp. were more infective against the *C. quinquefasciatus* larvae than the *Aspergillus* spp. as proved by their LC values (Table 1). The efficacy of *T. ajelloi* and *Chrysosporium* spp. were more effective than *Tolypocladium cylindrosporum* against 3rd-instar of *C. quinquefasciatus* [17]. The efficacies of *T. ajelloi* wet colonies (mycelia + spores) against *A. stephensi* and *C. quinquefasciatus* were more pathogenic against 3rd-instar than the other instars (1st, 2nd and 4th) is similar to the present investigation [9]. The LC₅₀ of *Metarhizium anisopliae* conidia suspension (1.97×10^4 conidia/ml) against *C. quinquefasciatus* is less effective than the LC₅₀ of *T. ajelloi* but nearly equal to the LC₅₀ of *C. pseudomerderium* and *Chrysosporium verruculatum* [18]. The LC₅₀ of *Penicillium citrinum* was 9.94×10^6 spores/ml against *Aedes aegypti* after 48 hours of exposure and is 9.3-fold higher than the present investigation [19]. The LC₅₀ of *C. lobatum* (0.13×10^3 conidia/ml) against the larvae of *A. stephensi* was less than the LC₅₀ of *C. pseudomerderium* (1.72×10^4 conidia/ml) and *C. verruculatum* (2.07×10^4 conidia/ml) against larvae of *C. quinquefasciatus* [15]. Sur et al. [5] recorded lower mortality (20–30%) in *A. stephensi* on day 7 against a concentration of 10^5 spore/ml of *A. flavus* is not comparable to the present investigation because of

differences in inoculation days. The differences in mortality in mosquito larvae occurred may be due to the high efficiency and specificity of the spores of fungi and variable of resistance in mosquito against fungal pathogenicity. All the fungi are developmental stage specific of the mosquito larvae and are more effective against the 3rd-instar larvae.

The surfaces of the fungus exposed larval body were uniformly covered by conidia except the head and siphon. Staining with cotton blue verified that the mycelium entered the haemolymph through the cuticle layers of the abdominal region (Fig. 2). The cuticle of the abdominal segments and lateral hairs were densely covered with conidia. The ventral brush and anal papillae of the larvae were also colonized with conidia and hyphae as in case of *T. cylindrosporum-Culex pipiens* infection [20].

An exotic species of *M. anisopliae* has a better chance of success against indigenous strain of mosquito larvae as the indigenous strain of *M. anisopliae* already stabilized a balance with the local mosquito larvae [21]. The floated spores of *Beauveria bassiana* caused two fold more mortality in the larvae of *Culex pipiens* than the submerged spores [22]. Therefore treating the habitat with exotic dry conidia is more practical possibility for biocontrol because it will remain floating for weeks and act more effectively. Thus in

Fig. 2 The 3rd-instar of *Culex quinquefasciatus* infected with conidia of *Trichophyton ajelloi*. Ct-Cuticle, Hy- Haemolymph, Ar-Abdominal region.

Table 1 LC₅₀ values with 95% fiducial limits in parentheses and probit equations of 3rd-instar *Culex quinquefasciatus* against seven fungal species

Fungal species	LC ₅₀ (spores/ml)	Probit equation
<i>Chrysosporium pseudomerderium</i> (EPL-903)	1.72×10^4 (1.46×10^4 – 1.93×10^4)	$3.500 + 0.354x$
<i>Chrysosporium verruculatum</i> (EPL-904)	2.07×10^4 (1.81×10^4 – 2.27×10^4)	$2.836 + 0.501x$
<i>Trichophyton ajelloi</i> (EPL-840)	4.08×10^3 (3.77×10^3 – 4.36×10^3)	$-2.315 + 2.026x$
<i>Aspergillus niger</i> (EPL-481)	2.17×10^7 (2.06×10^7 – 2.40×10^7)	$-4.622 + 1.311x$
<i>Aspergillus flavus</i> (EPL-457)	3.54×10^6 (3.14×10^6 – 3.71×10^6)	$-2.074 + 1.081x$
<i>Aspergillus fumigatus</i> (EPL-443)	1.07×10^8 (0.92×10^8 – 1.34×10^8)	$-2.836 + 0.977x$
<i>Penicillium chrysogenum</i> (EPL-450)	1.07×10^6 (0.84×10^6 – 1.22×10^6)	$-5.781 + 1.790x$

the present study we have used hydrophobic conidia of soil fungi instead of aquatic fungi. The results obtained in this study emphasize the variability in pathogenicity to mosquitoes among these fungi, which can be useful in designing future biological control programs. New bio-control agents are warranted to counteract the resistance developed in vectors to provide better alternatives for different environmental requirements. *Aspergillus spp.* produces mycotoxins and some of them are toxic, however, the breeding places of *C. quinquefasciatus* are unusable for humans viz., borrow pit, sewage drain and unused well. Therefore, the application of the fungi in the unused places is acceptable.

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