J. Commun. Dis. 37 (4) 2005 : 269-274

# Larvicidal Activity of the Fungus *Aphanomyces* (oomycetes : Saprolegniales) against *Culex quinquefasciatus*

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*(Received for publication : Feb. 2004)*


## ABSTRACT

**Several species of fungi are currently being considered for operational use in the microbial control of mosquito larvae. The oomycetous fungi are the prominent ones amongst them because of their ability to complete life cycle in water. During our studies on zoosporic fungi from riverine waters of Mula and Mutha flowing through Pune City, Maharashtra, India, *Aphanomyces laevis* de Bary (Oomycetes: Saprolegniales ) was isolated from polluted waters. After critical observations it was found to be mosquito larvicidal. Sporulating hemp seed cultures when inoculated under laboratory conditions revealed that it causes 80% mortality after seven days to *Culex quinquefasciatus* larvae. Laboratory assays were conducted to determine the effects of water quality on the ability of the isolate to infect mosquito larvae in varying degrees of pollution levels. In all the experiments, a non sexual strain of *Aphanomyces* (zoospores) was found to be the pathogenic agent for the *Culex* larvae.**


KEY WORDS: Aphanomyces, *C. Quinquesasciatus*

## INTRODUCTION

Mosquitoes are the vectors for some of the most deadly diseases known to man like malaria, filariasis, yellow fever and dengue etc. It has been responsible for a large number of deaths due to mosquito borne diseases especially in the third world countries like India. A highly effective way of controlling these diseases, obviously, would be control of the vector itself. The use of synthetic chemical insecticides for vector control is in decline due to high costs, the development of resistance in many target populations, and perceived risks to the environment and human health. Therefore biological control methods, especially microbial insecticides in particular are considered as alternatives to chemical insecticides because of their selective toxicity, ready decomposibility and safety.

The larval stage in the life cycle of mosquitoes is most vulnerable to control methods as they remain confined to the breeding place. This advantage of its restricted mobility is taken while formulating a biological control strategy. The most promising bio-control agents used so far against mosquito larvae are bacterial toxins from *Bacillus thuringensis israelensis* (B.t.i), *B. sphaericus* and *Lagenidium giganteum*, an oomycetous fungus. However, ingestion


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becomes pre-requisite in case of bacterial toxins to cause tissue destruction leading to death of mosquito larvae, on the other hand in case of *L. giganteum* the zoosporangia are too fragile for routine use in operational control programmes.[1,2] This is the reason, less attention has been paid to this group of biocides. Many of the fungal species are sensitive to ammonia, chloride and heavy metal ions. Thus they are not effective in highly polluted or brackish waters.[3]

During our studies on zoosporic fungi from riverine waters of Mula and Mutha flowing through Pune City, Maharashtra, India, *A. laevis* was isolated from polluted waters. The water chemistry of the sample from which the species was isolated from the water which was found to be polluted, has been given in Table 1. These rivers are less polluted in agricultural runoff areas, however, while passing through city domestic and industrial effluents make it highly polluted. Due to the organic effluent a larger surface area of the river is covered by water hyacinth which add to mosquito problem.

**Table 1 :Water analysis from which *A. laevis* was isolated**

| Parameters | Range |
|---|---|
| pH | 7.3 - 8.4 |
| Temperature (°C) | 21 - 26 |
| Dissolved Oxygen (ppm) | 0 - 1.5 |
| Free $CO_2$ (ppm) | 11.98 - 56.93 |
| Total Alkalinity (ppm) | 120 - 330 |
| Total Hardness (ppm) | 68 - 140 |
| Calcium (ppm) | 11.22 - 48.92 |
| Magnesium (ppm) | 2.43 - 23.86 |
| Chlorides (ppm) | 20.33 - 53.60 |
| Phosphates (ppm) | 0.65 - 1.68 |
| Electrical Conductivity (S / cm) | 274 - 745 |
| Total Dissolved Solids (ppm) | 178.1 - 484.2 |
| Dissolved Organic Matter (ppm) | > 50 |

Some oomycetous fungi such as *Leptolegnia caudata* and *A. laevis* were found as naturally occurring parasites of mosquito larvae, causing high mortality in *Anopheles culicifaecies* larvae. None of these is pathogenic to any crop and have no toxic effect on aquatic fauna and thus can be proposed as mosquito controlling agents, alternative to chemical insecticides[4].

Since, *A. laevis* is isolated from polluted river water, we felt it is worth determining its mosquito control potential in different degree of polluted water. With this view the present studies were conducted on three important mosquito vector species.

## MATERIALS AND METHODS

### Study area

Pune is situated on the bank of rivers Mula and Mutha between 18° 31' N Lat. & 73° 51'E Long. The rivers originate along the eastern flank of the Western Ghats of Maharashtra. Ten sampling stations were established along the course of rivers (4 on river Mula, 4 on river Mutha and 2 on Mula-Mutha). These stations were selected in relation with the point sources of pollution such as domestic effluents discharging outfalls, the industrial effluent release points and the non point sources such as agricultural runoff areas. Composite surface water samples were aseptically collected in polythene containers at monthly intervals in the morning to evaluate the spatio-temporal trends. The samples were subjected to physico-chemical analysis which included the important parameters like pH, temperature, dissolved oxygen.(D.O.), Ca, free $CO_2$, total alkalinity, total hardness, chlorides, dissolved organic matter (D.O.M.), phosphates. For all the procedures, Standard Methods defined by American Public Health Association[5] was

followed.

**Isolation of Aquatic Fungi**

From all the water samples aquatic fungi were isolated using baiting technique.[6] Boiled hemp seeds (*Cannabis sativa*), sterilized grass leaves and insect legs were used as baits. Identification of the fungus isolated in the laboratory was done according to key defined by Scott.[7]

**Pathogenecity Tests**

Laboratory bioassays were performed by exposing mosquito larvae to the fungal spores. Mosquitoes of all the three species i.e. *C. quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* used for the bioassays were taken from the laboratory colonies at National Institute of Virology, Pune maintained at insectory at 28 ± 2°c, 85 ± 5 % RH. The assays were conducted in 50 ml of distilled water in glass jars containing thirty II- instar larvae to study the pathogenecity of the fungus. A pure hemp seed colony of the fungus was kept in sterile distilled water for zoosporogenesis. Two, 72 hrs old hemp seed colonies of the test fungus were added to each jar. A control without the inoculum was also run simultaneously for comparison. Mortality was counted after 24 hrs upto the emergence of the adult. The pathogen associated with the dead larvae was re-isolated and compared with test fungus.

**Preparation of Zoospore Suspension**

Effective zoospore suspension was prepared according to method shown by Sullia.[8] The relationship between zoospore concentration and larval mortality was determined using zoospore suspension. Two, Peptone Yeast Extract Glucose agar plates were inoculated with the fungus by placing bits of inoculum at equidistant spots and incubated at 28°C for four days. The medium with the mycelium was then cut into thin strips with a sterile scalpel and transferred into 500 ml of sterile sporulation medium containing 0.25 mM of $CaCl_2$ and 0.25 mM of KCl. Two changes of sporulation medium were given to wash off all the nutrients in the agar strips. The mycelium was then incubated overnight in 500 ml of sporulation medium at 28°C on a shaker. The zoospore suspension was then filtered through sterile membrane to separate the zoospores from the mycelia. The spore concentration ranging from of $3.5x10^5$ to $6.0x10^5$ / ml was obtained by this method.

An another set of experiment was run to see the effect of extra cellular toxins and to check which is the killing component. Germinated zoospores are filter sterilized. The entire suspension was centrifuged at high speed (10,000g for 10 min). Thirty II- instar larvae were released in the supernatant. A control without the inoculum was used as control and daily mortalities were recorded.

## RESULTS

The site from which *A. laevis* is isolated, was highly polluted since very low values of D.O. and high values of D.O.M. were recorded (Table 1). This profile of low D.O. and high D.O.M is the result of rapid deoxygenation of water as evidenced by anaerobic conditions. This may be due to joining of some low order streams carrying domestic and sewage wastes to the river channel. Among the three species of mosquitoes are tested, it was found interesting that this fungus which was isolated from polluted water was found to cause heavy mortality in *C. quinquefasciatus* species. Such heavy organically polluted habitat is preferred by *C. quinquefasciatus* for breeding.[9] Females of *C. quinquefasciatus* lay eggs only after taking a blood meal, and the larvae are found in a wide variety of artificial containers in just about any water container found near human habitat and various types of stagnant water such as ditches, gutters and ground pools.[10]

**Table 2 : Susceptibility of three species of mosquitoes to *A. laevis* zoospores[1]**

(Average data of three replicates ± standard deviation)

| Species | Cumulative Percentage Mortality[2] | % Pupal Emergence | % Adult Emergence |
|---|---|---|---|
| *C. quinquefasciatus* (Test) | 80.48 ± 27.95 | 19.52 | 19.52 |
| *C. quinquefasciatus* (Control) | 8.88 ±1.92 | 91.12 | 91.12 |
| *A. aegypti* (Test) | 0 | 100 | 100 |
| *A. aegypti* (Control) | 0 | 100 | 100 |
| *A. stephensi* (Test) | 0 | 100 | 100 |
| *A. stephensi* (Control) | 0 | 100 | 100 |

Each assay was performed in triplicate using 30 II-instar larvae, cumulative percent mortality after seven days exposure at room temperature.

.Corrected mortality (Abbott's correction).

**Table 3: Percent Mortality of *C. quinquefasciatus* larvae to various concentrations of *A. laevis* zoospores.**

| Zoospore conc. (spores / ml) | $5.0 \times 10^5$ | $1.0 \times 10^6$ | $1.5 \times 10^6$ | $2.0 \times 10^6$ |
|---|---|---|---|---|
| Cumulative Percentage Mortality* | 30 | 36.66 | 53.33 | 80 |

* Assay was performed using 30 II-instar larvae, cumulative percent mortality after seven days exposure at room temperature.

**Table 4 : Effect of fungus on mosquito in water derived from station 1 and 2 and water chemistry parameters of these two water samples[1]**

(Average data of two replicates ± standard deviation)

| Parameters | Station 1 | Station 2 |
|---|---|---|
| Percent Mortality | 76.48 ± 8.00 | 59.51 ±1.68 |
| pH | 7.38 | 8.24 |
| Temperature °C | 24 | 25.2 |
| Dissolved Oxygen (ppm) | 6.1 | 0 |
| Free $CO_2$ (ppm) | 2.99 | 120.75 |
| Total Alkalinity (ppm) | 47 | 193 |
| Total Hardness (ppm) | 43 | 274 |
| Calcium (ppm) | 16.83 | 66.53 |
| Chlorides (ppm) | 8.98 | 44.41 |
| Phosphates (ppm) | 0.2 | 1.2 |
| E.C. ($\mu^S$/cm) | 270 | 825 |
| Dissolved Organic Matter (ppm) | <20 | >50 |

[1] Each assay was performed in duplicate using 30 II-instar larvae, cumulative percent mortality after seven days exposure at room temperature.

Among other two species, *Aedes aegypti* also showed some susceptibility to the fungus in term of extending larval duration by one or two days (Table 2). The mortality of *C. quinquefasciatus* was found to be associated with the dose of zoospores (Table 3).

To determine effectiveness of this fungus in different degrees of polluted water, naturally polluted water from station 1 and station 2 was drawn and brought to laboratory. This was filtered through the milipore to remove existence of naturally occurring zoospores. Bioassays were performed and simultaneously water was analyzed for above mentioned parameters to assess the degree of pollution in these stations. Results showed that the fungus was effective in polluted water of station 1, however even at higher degree of pollution at station 2, it could cause higher mortality in *C. quinquefasciatus* larvae (Table 4).

## DISCUSSION

Infection occurs when zoospores encyst on the larval cuticle and form a germ tube which subsequently develops and penetrates into the host, leading to destruction of tissues. The encystment of zoospores on the larval cuticle and mechanism of damage was confirmed using Methylene Blue Test.[11] Penetration involves both mechanical and chemical activities. Destruction of protective larval cuticle due to different cuticle degrading enzymes and their role in causing mortality in mosquito larvae is well known.[12] Once fungi invade the haemocoel, the host may be killed by fungal growth leading to nutrient exhaustion. Extra cellular toxins are not secreted by the zoospores. This is supported by the observation that mortality was not observed when the larvae are released in the supernatant. In all the bioassay experiments, the test fungus showed mortality only in the larvae of *C. quinquefasciatus*. It was interesting to note that the three species of mosquito tested for pathogenecity, the test fungus is showing host specificity to the *Culex* larvae. Therefore, further studies were carried out on *C. quinquefasciatus* larvae. In all the experiments larval mortality was observed after three days. It appears that the given dose of zoospores was lethal for *Culex* larvae, but it took two days for fungus to get establish in the body of mosquito larvae and to produce mortality.

In the case of *A. aegypti* mortality was not observed but there was some retardation of growth in larvae and adults which emerged from the larvae exposed to zoospores were very weak. The absence of mortality in *A. aegypti* larvae showed it is non target organism but delay in emergence of adults for the exposed larvae suggest that this fungus must have invaded the body but did not establish itself to the level which can cause mortality in the larvae. Invasion of non target species and low level of development may be the mechanism for their persistence in nature. Though *A. aegypti* do not breed in polluted water, there is possibility that it might be maintained in some other species of mosquito or any other arthropod.

It has been observed that physico-chemical parameters have affected the infection rate and subsequent mortality. Higher infection rates at station 1 were recorded because of low levels of organic pollution as evident by high D.O. values coupled with low free $CO_2$ and D.O.M. values. Alkalinity and Hardness levels were considered too low to reduce infection rates. This is in accordance with the results obtained by Hallmon *et al.*[13] pH of the water was found to have no significant effect on the zoosporogenesis. With increasing organic pollution, there was a decrease in the infection rate and mortality of larvae. This may be because of competition to mosquito larvae for fungus from other benthic feeders. The effects of polluted water are to reduce the

sporogenesis. Therefore, even if larvae are infected its transfer of infection to new hosts will largely depend upon the water parameters. Thus, status of the fungus as a self perpetuating agent needs to be critically evaluated. A larger surface area of these two rivers are covered by water hyacinths. Due to this a high prevalence of Mansonoids species of mosquitoes have been recorded in many areas. It would interesting to determine if this fungus is effective against Mansonia mosquitoes, since it also develops in polluted water, it can be used in this ecosystem for the control of mosquitoes.

## ACKNOWLEDGMENT

Senior author is thankful to the Principal, Modern College for providing necessary laboratory facilities. Thanks are also due to the Head, Department of Environmental Sciences, University of Pune, and the Director, National Institute of Virology, where this work was conducted.

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