PERSISTENCE OF THE MOSQUITO PATHOGENIC FUNGUS *CULICINOMYCES* IN ARTIFICIAL AQUATIC ENVIRONMENTS

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ABSTRACT. The ability of *Culicinomyces* conidia to persist and remain pathogenic to two mosquito species was studied in different aquatic environments in the laboratory. At 14°C conidia persisted and remained pathogenic to *Aedes aegypti* larvae for up to 112 days whereas at 25°C conidia were pathogenic for a maximum of 28 days. Mortalities of *Culex quinquefasciatus* larvae were lower than *Aedes aegypti* at 25°C. This was attributed to differences in feeding behavior. The short term effect of substrate type was minimal but larval mortality in cups containing coarse sand sediment fell at a faster rate than finer mineral sediments. This phenomenon may be due to conidia falling into the larger pore spaces and becoming inaccessible to mosquito larvae.

INTRODUCTION

The Australian isolate of the mosquito pathogenic fungus *Culicinomyces clavisporus* Couch, Romney and Rao has undergone extensive laboratory and field study since its discovery in 1972 (Sweeney et al. 1973, Sweeney and Panter 1977, Sweeney 1981b). The work has progressed to the field evaluation stage, with a large scale field trial showing the fungus successful in controlling *Culex annulirostris* Skuse larvae breeding in freshwater ground pools (Sweeney et al. 1983).

The potential of a biocontrol agent to persist in the habitat of a target species must be assessed before large scale use can be implemented (Smith 1973). Sweeney has outlined the prospects for the field use of *Culicinomyces* as a mosquito control agent, reported the lack of persistence of the fungus in natural sites and suggested that more research was required to investigate its ability to persist in different environments (Sweeney 1981b, 1981c; Sweeney et al. 1983).

Three concerns were raised in earlier studies:

(1) In the laboratory the conidia of *Culicinomyces* lose their viability and infectivity to mosquito larvae after several weeks in distilled water at 25°C, at 4°C they remain viable and pathogenic for up to 4 months, and when stored in distilled water at −70°C they show no significant decrease in potency after 6 months (C. Panter, unpublished data; Sweeney 1981a).
(2) The conidia of *Culicinomyces* settle to the bottom of a body of water at a rate of approximately 8cm/day (Sweeney 1981b) after application to the surface and it has been proposed that this may make them less accessible to non-bottom feeding mosquito species (Cooper and Sweeney 1982, Russell et al. 1983).
(3) Some mosquito species often "graze" the bottom substrate for food (Christophers 1960) and the physical characteristics of the substrate may be important in the ingestion of sunken conidia by larvae.

Therefore, the effect of temperature on the ability of conidia to persist and remain infective, the influence of feeding behavior of the mosquito larvae on infection and the influence of substrate on the ability of conidia to persist and remain accessible to larvae were investigated using artificial aquatic environments.

MATERIALS AND METHODS

Six different types of aquatic environment were prepared in the laboratory in 250 ml plastic cups (9 cm diam, 6 cm high). These were:

(1) Coarse sand: 100 g of sand (particle size range, 1.2–1.4 mm), sterilized by heating at 160°C for 4 hr in an oven, and 100 ml of distilled water.
(2) Medium sand: 100 g of sterile sand (0.25–0.50 mm) and 100 ml of distilled water.
(3) Fine sand: 100 g of sterile sand (0.10–0.15 mm) and 100 ml of distilled water.
(4) Leaf material: This environment consisted of dead *Eucalyptus* sp. leaves collected in the field. The leaf material, in various stages of decomposition, was pasteurized by heating to 70°C for 15 minutes to kill any fungal pathogens that may have been present. Each cup contained five leaves and 100 ml of distilled water.
(5) Twig material: Five pasteurized field collected dead twigs (approx. 5 cm long, 2–5 mm diam) and 100 ml of distilled water.
(6) Sediment free: 100 ml of distilled water to act as a control environment.

The fungal inoculum was grown in submerged culture (Oxoid Lab. Lemco Nutrient Broth amended with 100 ppm streptomycin and 20 ppm neomycin), conidia were harvested and stored at −70°C (Sweeney 1981a). A spore suspension was prepared and numbers estimated using an Improved "Neubauer" haemocytometer. A bioassay of the inoculum gave an LC_{50} of 8×10^2 conidia/ml against first instar *Aedes aegypti* (Linnaeus) larvae following the method of Cooper and

Sweeney (1982) which recorded mortality after 4 days exposure.

One hundred and eighty cups of each environment (1–6) were held at 25 ± 1°C in an air-conditioned laboratory and 60 cups of environments 1, 4 and 6 were held at 14 ± 1°C in a Labec incubator. Of the cups held at 25°C, 60 of each environment were inoculated with sufficient conidia to yield a dose rate of 10^5 conidia/ml. Another 60 were inoculated with 10^3 conidia/ml and the remaining 60 cups were left as fungus free controls. These dose rates were also applied to cups held at 14°C, with 20 cups of each environment receiving 10^5 conidia/ml, 20 receiving 10^3 conidia/ml and 20 left as fungus free controls.

Assessment of the ability of *Culicinomyces* conidia to remain accessible and pathogenic to mosquito larvae was determined by bioassay challenge. When challenges were run for cups held at 25°C, ten first instar larvae were added to each of three replicate cups from each environmental treatment. For cups held at 14°C ten first instar larvae were added to duplicate cups. All challenges were conducted in the laboratory at 25°C.

Using *Ae. aegypti* larvae, the challenges of cups held at 25°C were run at 8, 14, 21, 29 and 43 days after inoculation, and for those held at 14°C at 8, 14, 21, 36, 42, 60, 77, 91 and 112 days after inoculation. *Culex quinquefasciatus* Say larvae were used 7, 14 and 28 days after inoculation for cups held at 25°C.

Larvae were fed 0.2 ml of autoclaved yeast suspension (5% w/v) per cup on day zero, 2 and 4 of the challenge. The mortality in each cup was recorded after 7 days by counting the survivors, and was adjusted for control mortality using Abbott's formula (Finney 1952). Statistical significance was assessed using chi-square tests.

RESULTS AND DISCUSSION

The control mortalities averaged over all the environments were: 9% for *Ae. aegypti* at 14°C, 5% for *Ae. aegypti* at 25°C and 13% for *Cx. quinquefasciatus* at 25°C. There was no evidence of heterogeneity of mortality between replicates, thus the replicates could be combined to produce estimates of mortality. For the purpose of tests for significance, the number of larvae that died has been treated as a binomial variable with sample sizes of 30 at 25°C and 20 at 14°C. These moderate sample sizes were combined with further replication over time to increase precision.

The ability of *Culicinomyces* applied at 10^5 conidia/ml to persist and remain pathogenic to *Ae. aegypti* larvae in the environments held at 25°C is shown in Fig. 1A. It can be seen that 100% mortality was observed in all environments at day 8, and in all but one environment at day 14. After 14 days the mortality of larvae in subsequent challenges decreased until zero corrected mortality was observed in all environments after 43 days. Mortality data for cups inoculated with 10^3 conidia/ml is not shown in Figure 1A as the mortality in these cups did not differ from the control cups after 8 days.

The mortality of *Ae. aegypti* larvae in cups held at 14°C is shown in Fig. 2. The mortality of larvae challenged with 10^5 conidia/ml remained higher than 80% in all environments for 42

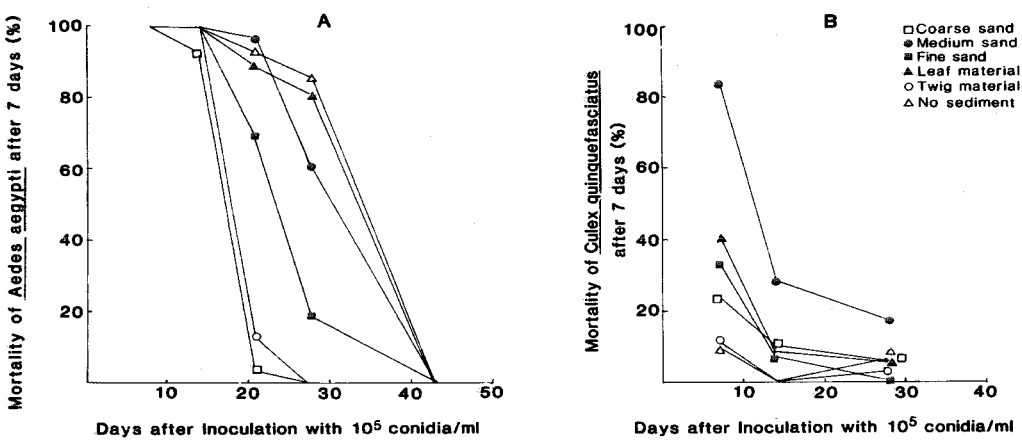

Fig. 1. Persistence of *Culicinomyces* conidia at 25°C in aquatic environments.

days, and the mortality of larvae added to leaf containing and sediment free cups remained greater than 80% for up to 112 days after inoculation. The mortality of larvae in the coarse sand environment declined after 42 days to be less than 10% at 91 days, with larval mortality significantly lower than in sediment free cups at 60 days ($p < 0.0l$), 77 days ($p < 0.025$), 91 days ($p < 0.01$) and 112 days ($p < 0.01$).

The mortality of larvae challenged with 10^3 conidia/ml held at 14°C is also shown in Fig. 2. After 8 days the mortality observed was only slightly greater than 50% which was to be expected since this dose was close to the LC_{50} value determined for these conidia. Larval mortality fell to below 10% in all cups after 21 days and remained very low for up to 90 days.

This temperature response was shown not to be constant for the two mosquito species. Figure 1B shows the results when the mortality of *Cx. quinquefasciatus* larvae was measured after challenge with 10^5 conidia/ml under conditions similar to the *Ae. aegypti* trial (Fig. 1A). *Culex quinquefasciatus* larval mortality was low after 7 days, except in cups with medium sand, and by 14 days was less than 30% for all environments, significantly lower than the equivalent results for *Ae. aegypti*. The difference in mortality between the two species was significant ($p < 0.01$) in all environments except the medium sand, at 7 days, and in all environments at 14 days.

The lack of significant differences in susceptibility between these two species was demonstrated in laboratory bioassays in sediment free cups by Cooper and Sweeney (1982), and the differences in feeding behavior of the two species (Christophers 1960) could possibly explain these results. Although neither species displays such respective behavior exclusively, the bottom foraging nature of *Ae. aegypti* could result in the ingestion of a larger number of conidia, after these had settled to the bottom, than the filter feeding activities of *Cx. quinquefasciatus*.

Possibly because of this feeding behavior, any effects of bottom substrate on larval mortality of *Cx. quinquefascuatus* (Fig. 1B) are difficult to discern, and no conclusions could really be drawn in this regard. However, for *Ae. aegypti* (Figs. 1A, 2) a trend was observed in the trials run at both 25°C and 14°C in the cups inoculated with 10^5 conidia/ml. Mortality at 14°C fell at a faster rate for coarse sand than for either leaf material or for no sediment. Similar results were obtained at 25°C with the medium sand, fine sand and twig material showing intermediate mortalities.

The common mode of infection in susceptible larvae is that conidia are ingested and become attached to the interior wall of the foregut. Subsequent germination and hyphal growth within the haemocoel kills the larva (Sweeney 1975). The reduced mortality observed in coarse sand cups over time could be due to fewer conidia being available to mosquito larvae, as the conidia may become incorporated between the sand particles and thus inaccessible to grazing larvae.

The issue may not be so simple, however, as the effects of the bottom activity of larvae in resuspending settled conidia may need to be taken into account, as would the relative depth of the water itself, and the viability of the conidia, but unfortunately these considerations were not included in the investigation.

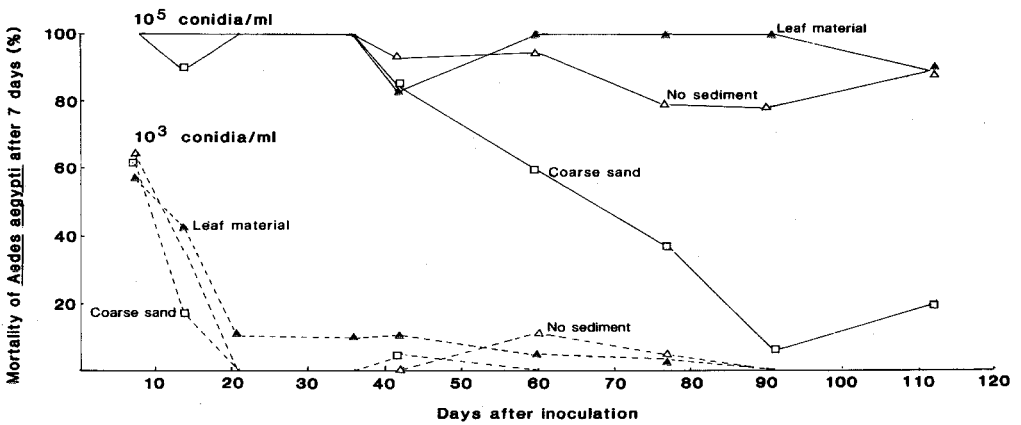

Fig. 2. Persistence of *Culicinomyces* conidia at 14°C in aquatic environments.

CONCLUSIONS

This study has confirmed that the ability of *Culicinomyces* conidia to persist is prolonged by reduced temperatures in the laboratory. Future work is required to assess the effect of water temperature on the ability of the fungus to persist in natural field habitats.

The feeding behavior of the mosquito species tested was shown to be important, and with the nature of the bottom sediment, may significantly affect the mortality rates of target species. This supports earlier studies (Russell et al. 1983) which indicated that the sinking of the conidia may be advantageous for use against bottom feeders, such as *Aedes* larvae, but it may be necessary to formulate them so they remain close to the surface for control of *Anopheles* and *Culex* larvae.

Overall, this study has shown the limited potential of *Culicinomyces* conidia to persist under some conditions in aquatic environments in the laboratory. Future studies will investigate persistence in natural environments.

ACKNOWLEDGMENTS

Financial support was provided by the National Health and Medical Research Council of Australia and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We thank Mr. S. Mather for technical assistance, Assoc. Prof. G. Berry for statistical advice and Dr. A. W. Sweeney for reading the final draft.

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MARCO ENRICO GIGLIOLI FUND

At the AMCA Board of Directors Meeting in Toronto, Canada in March 1984, a memorial fund was established in memory of Dr. Marco Giglioli who passed away recently after a long illness. The monies from this fund will help to support the publication of papers by foreign authors who cannot obtain U. S. funds. Donations should be sent to the AMCA Central Office, 5545 East Shields Avenue, Fresno, CA 93727. Names of contributors will be published annually in *Mosquito News*.