STUDIES ON BIOASSAY OF THE ENTOMOPATHOGENIC HYPHOMYCETE FUNGUS *TOLYPOCLADIUM CYLINDROSPORUM* IN MOSQUITOES

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ABSTRACT. Larvae of *Aedes aegypti*, *Ae. vexans* and *Culiseta inornata* were used to study the pathogenicity of the hyphomycete fungus *Tolypocladium cylindrosporum*. Experiments consisted of continuous exposure of second-instar larvae to 5 concentrations of conidia at 10, 15, 20 and 25°C. The 3 test species were susceptible at all temperatures tested. There was generally a direct relationship between dosage and pathogenicity in the host population, however linear relationships between probit mortality at 10 days and log dosage occurred in only 11 of 37 assays performed. In these 11 assays, median lethal concentrations were in the order of 10^4–10^5 conidia/ml. Median lethal times were between 3 and 14 days. It was concluded that the present bioassay method for *T. cylindrosporum* is inadequate and needs major improvement.

INTRODUCTION

Difficulties associated with bioassay of insect pathogens are well documented (Bucher and Morse 1963, Burges and Thompson 1971, Huber and Hughes 1984). To date only a few assay systems have been developed for fungi as there are many problems associated with their bioassay; the greatest being inability to deliver infectious propagules to the assay host in a standardized manner. The usual route of fungal infection is *via* the exocuticle, however, in the mosquito pathogenic fungus *Culicinomyces clavisporus* Couch, Romney and Rao, the primary route of infection is through the foregut and hindgut following ingestion of the conidia (Sweeney 1975). Sweeney (1976) was able to obtain adequate results with *C. clavisporus* based on the highly successful mosquito bioassay method for chemical insecticides (Brown and Pal 1971) and *Bacillus thuringiensis israelensis* (McLaughlin et al. 1984); namely exposure of mosquito larvae to different concentrations of test material in the rearing medium. Although results were highly variable, the log dose, probit mortality regressions were generally linear.

Tolypocladium cylindrosporum Gams is a potential microbial control agent of mosquitoes (Soares et al. 1979, Weiser and Pillai 1981). Infection sites are through the external cuticle, pharynx and midgut and preliminary evaluation of dosage-responses indicated that probit mortality regressions were generally linear (Soares 1982).

This paper reports on dose-mortality assays conducted to quantify the pathogenicity of *T. cylindrosporum* conidia at different temperatures using larvae of *Aedes aegypti* (Linn.), *Ae. vexans* (Meigen) and *Culiseta inornata* (Williston).

MATERIALS AND METHODS

Mosquito colonies. A Florida strain of *Ae. aegypti* was obtained from the Insects Affecting Man and Animals Research Laboratory, Gainesville, Florida and a colony of *Cs. inornata* was established from field-collected larvae and adults from the Edmonton area. All *Ae. vexans* larvae used were field-collected from the Devon, Alberta area.

Inoculum preparation. Californian isolates of *T. cylindrosporum* [TC3 = INRA 3 (Institut National Recherche Agronomique, La Minière France) = UAMH 4561 (University of Alberta Microfungus Collection and Herbarium, Edmonton, Canada); TC4 = INRA 4 = UAMH 5002] as well as isolate TC3 passaged through mosquito larvae 6 (TC3S6), 13 (TC3S13), and 18 (TC3S18) times (Goettel 1987a) were cultured for two weeks at 20°C on 50 ml Pablum™ mixed cereal agar (Padhye et al. 1973) or potato dextrose agar supplemented with 60 μg/ml penicillin and 30 μg/ml streptomycin (PDA-SP) in 200 ml culture flasks. Conidial suspensions were obtained by adding 100 ml of sterile distilled water to each culture flask and shaking for several minutes. Conidial counts were made using an improved Neubauer hemocytometer. Appropriate serial dilutions were made in Bates' medium S (McLintock 1952) to give final concentrations of 1×10^3, 10^4, 10^5, 10^6 and, on several occasions, 10^7 conidia/ml.

Conidial viability was determined by spreading 0.1 ml of a 1×10^6 conidia/ml suspension onto the surface of PDA-SP in a standard Petri dish. Following incubation at 25°C for 24 hr, the numbers of viable and non-viable conidia were counted on the plate surface under phase contrast at a magnification of 200×. Conidia were

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Table 1. Mortality rate and proportion of dead larvae with diagnosed mycosis after exposure to different concentrations of *Tolypocladium cylindrosporum* conidia for 20 days at different temperatures.[1]

Mosquito species	Temp °C	No. of assays[2]	% mortality (proportion infected)[3] at each dose[4]					
			0	10^3	10^4	10^5	10^6	10^7
Cs. inornata	20	3	48 (0)	69 (6)	73 (28)	90 (60)	97 (79)[5]	nd[6]
	15	3	28 (0)	19 (0)	28 (19)	51 (64)	67 (72)[5]	nd[6]
	10	1	15 (0)	15 (0)	8 (0)	12 (0)	72 (41)	nd[6]
Ae. vexans	25	1	92 (0)	93 (0)	95 (0)	97 (5)	100 (33)	100 (18)
	20	2	60 (0)	61 (16)	65 (18)	70 (42)	97 (59)	98 (59)[5]
	15	1	70 (0)	73 (0)	68 (0)	86 (36)	100 (50)	98 (39)
	10	1	50 (0)	50 (0)	53 (0)	92 (5)	98 (29)	100 (15)
Ae. aegypti	25	2	2 (0)	20 (25)	22 (55)	41 (67)	67 (58)	83 (30)[5]
	20	6	9 (0)	16 (34)	26 (57)	63 (56)	93 (54)	100 (78)[5]
	15	2	26 (0)	24 (7)	47 (25)	72 (62)	99 (47)	97 (38)[5]
	10	2	100 (0)[5]	100 (0)[5]	90 (11)[5]	97 (31)	99 (31)	100 (82)[5]

[1] Pooled data. Isolate TC3.
[2] Each assay consisted of continuous exposure of 60 second-instar larvae per dose.
[3] Proportion of deads infected = no. with mycosis/total no. dead × 100.
[4] Conidia/ml.
[5] Results of one assay.
[6] Not done.

considered viable if they swelled to a "barbell" or "peanut" shape. This swelling occurs just prior to the formation of a germ tube (Goettel 1987b[2]). Three replicates were prepared for each assay. Counts were made of 5 fields of view per plate. Viabilities were always above 70% and in most cases, over 85%.

Assay method. Batches of 20 second-instar larvae were added to 200 ml of each conidial concentration in 500 ml plastic containers (7 cm high × 11 cm wide; approx 3 cm water depth). A pinch of ground Tetramin® fish food was sprinkled onto the surface of each container for larval food and was subsequently added as required. All containers were covered with sheets of glass to minimize evaporation and were kept in incubators at either 10, 15, 20 or 25°C. *Aedes aegypti* larvae were kept at a photoperiod of 12/12 while *Ae. vexans* and *Cs. inornata* were kept at 16/8 (L/D). Distilled water was added periodically to compensate for evaporation. Pupae were removed daily and were placed singly into multiple-chambered trays (15 ml capacity/cell) until emergence. Adults were kept in the culture tray for at least 48 hr after emergence. Dead insects were removed daily, stored at 4°C for 24 to 48 hr and were then examined microscopically for mycosis i.e., presence of hyphae in the hemocoel. Within 48 hr of death, infected adults could be easily recognised by the appearance of a white cotton-like growth of the fungus on cadavers. Each assay consisted of 3 replicates at each of 4 or 5 concentrations and a control. Thirty-seven bioassays consisting of different mosquito/temperature/isolate combinations were carried out.

Dose and time-mortality results of each assay were subjected to probit analysis (probit mortality and log dose or time) (Finney 1971) using the computer program of SAS Institute Inc., Cary, NC. All mortalities were adjusted for control mortality using Abbott's (1925) formula. Time-infection mortality responses were also subjected to probit analysis (probit infection mortality as percent of total number infected and log time) to obtain estimated ST_{50}s (survival time for 50% of total number infected). Statistically significant differences were judged by mutually exclusive 95% fiducial limits.

Infection rate data were analyzed by one-way analysis of variance using angular transformation of proportions infected and log transformed numbers of individuals infected. These were further analyzed using Scheffé's test at the 95% level of significance (Sokal and Rohlf 1969).

RESULTS

All 3 mosquito species were found susceptible to the fungus at all temperatures tested (Table 1). *Culiseta inornata* is a new experimental host. Mosquitoes succumbed to the fungus at all developmental stages tested, with 90% of the total infected in all assays (n = 1,584) dying as larvae, 9% as pupae, and 1% as adults. Control mortalities in *Ae. vexans* and *Cs. inornata* were high. *Aedes aegypti* proved to be a better bioassay organism because of lower control mortalities, but control mortality was 100% in this species at 10°C.

[2] Goettel, M. S. 1987b. Studies on microbial control of mosquitoes in central Alberta with emphasis on the hyphomycete *Tolypocladium cylindrosporum*. Ph.D. Thesis, University of Alberta, Edmonton. 198 pp.

Examples of time-dose-mortality responses obtained for each species are given in Figs. 1–3. There was generally a direct relationship between dosage of conidia and pathogenicity in the host population, however, there was much variability. In some instances higher mortalities were obtained at a given dose than at the next higher dose (i.e., see Fig. 2). Eleven of the 37 bioassays performed showed linear relationships between probit mortality at 10 days and log dose (Table 2). Mortalities taken at 20 days were even less amenable to probit analysis as in most of these, mortalities of 100% were obtained at the highest concentrations, while at the lowest concentration the mortalities were lower or equal to the control mortality. In such cases, there were only two points on which to base the dose-mortality regression line. In the assays where probit analysis was possible on 15 and 20 day mortality, the LC_{50}s did not differ significantly from those at day 10.

There were significant differences in the LT_{50}s between replicate assays (Table 3). For instance, for *Ae. aegypti* with isolate TC3 at 20°C the variance comprised large, significant between-assay components (5.99, 7.72) and small, within assay components (0.11, 0.06) (for 10^5 and 10^6 conidia/ml respectively), thereby

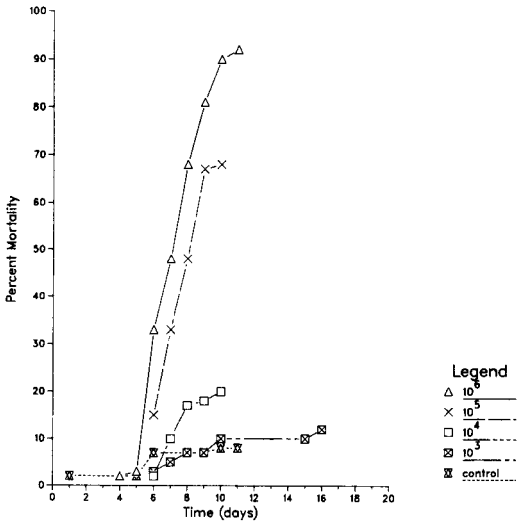

Fig. 1. Cumulative mortality of *Aedes aegypti* exposed to varying concentrations of *Tolypocladium cylindrosporum* (TC3) conidia at 20°C. Sixty second-instar larvae were exposed at each concentration (assay no. 18).

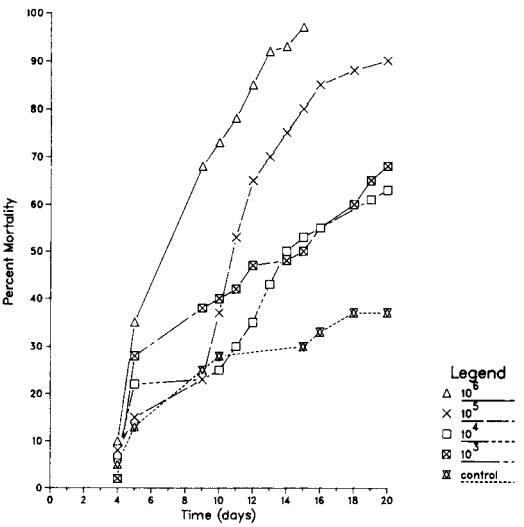

Fig. 2. Cumulative mortality of *Culiseta inornata* exposed to varying concentrations of *Tolypocladium cylindrosporum* (TC3) conidia at 20°C. Sixty second-instar larvae were exposed at each concentration (assay no. 10).

Table 2. Probit analysis of dose-mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum*.[1]

Mosquito species	Fungus isolate	Temp. °C	Assay[2] no.	LC_{50} ($\times 10^4$ conidia/ml)	95% fiducial limits	Chi square (df)	H[3]	Slope ± SE
Ae. vexans	TC3	20	18	2.8	1.0–5.5	1.05 (2)	+	1.14 ± 0.218
Ae. aegypti	TC3	25	15	67.6	42.3–108.5	5.00 (3)	+	1.01 ± 0.114
			21	15.2	6.6–46.8	3.11 (2)	+	0.49 ± 0.086
		20	18	6.5	3.9–10.4	2.02 (2)	+	1.17 ± 0.159
			21	3.4	0.2–39.5	4.95 (2)	−	0.98 ± 0.177
		15	15	44.5	23.8–81.2	5.75 (3)	+	0.78 ± 0.105
	TC4	20	16	5.6	3.6–8.5	3.96 (2)	+	1.41 ± 0.181
	TC3S6	20	21	5.4	3.8–7.9	2.00 (2)	+	1.44 ± 0.154
	TC3S13	20	16	1.2	0.7–1.9	1.63 (2)	+	1.16 ± 0.147
	TC3S18	20	20	0.7	0.5–1.0	0.18 (1)	+	1.36 ± 0.165
			21	6.7	4.6–9.7	1.10 (1)	+	1.54 ± 0.182

[1] Mortalities were taken after 10 days of continuous exposure of second-instar larvae and were corrected for control mortality using Abbott's formula.

[2] Each assay consisted of four dose levels with 60 larvae/dose.

[3] Within assay homogeneity.

Table 3. Probit analysis of time-mortality responses of mosquitoes exposed to conidia of *Tolypocladium cylindrosporum*.[1]

Mosquito species	Temp. °C	Assay[2] no.	LT_{50} (1×10^5 conidia/ml)	95% limits[3]	LT_{50} (1×10^6 conidia/ml)	95% limits[3]	% control mortality[4]
Ae. vexans	20	18	7.2	7.0–7.5	6.2	6.0–6.4	43
	10	14	13.5	13.1–13.8	13.4	13.0–13.8	33
Cs. inornata	20	07	13.6	12.6–15.0	nd[5]	—	17
		10	12.7	12.2–13.2	8.6	8.0–9.1	27
		11	10.0	9.5–10.6	nd[5]	—	23
Weighted Mean[6]:			12.1	7.5–16.8			
	15	10	mort. <50%		13.8	13.3–14.3	20
Ae. aegypti	25	15	mort. <50%		7.4	7.0–7.8	2
		21	mort. <50%		6.5	6.0–7.0	3
	20	12	mort. <50%		7.6	7.2–7.9	8
		15	10.1	9.6–10.5	6.8	6.1–7.4	10
		16	14.0	13.1–15.3	11.9	11.2–12.7	2
		18	8.4	8.0–8.9	7.4	7.1–7.7	8
		20	mort. <50%		3.3	2.6–3.7	8
		21	9.6	9.2–10.0	6.0	5.6–6.3	2
Weighted Mean[6]:			10.5	6.5–14.4	7.1	4.2–10.1	
	15	12	mort. <50%		8.7	8.0–9.2	20
		15	11.9	11.6–12.2	7.9	6.7–8.8	7

[1] Isolate TC3. Mortalities were corrected for control mortalities on each day using Abbott's formula.
[2] Each assay consisted of continuous exposure of 60 second-instar larvae per dose.
[3] Fiducial limits.
[4] Control mortality at 10 days.
[5] Not done.
[6] Calculated according to Bliss (1952).

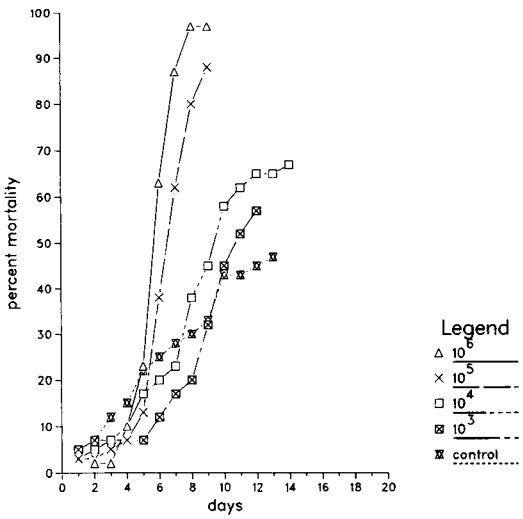

Fig. 3. Cumulative mortality of *Aedes vexans* exposed to varying concentrations of *Tolypocladium cylindrosporum* (TC3) conidia at 20°C. Sixty second-instar larvae were exposed at each concentration (assay no. 18).

contributing to the very wide 95% fiducial limits of the weighted means. The LT_{50}s increased with a decrease in temperature for *Ae. vexans* and *Cs. inornata*, however this effect was not as apparent in *Ae. aegypti*. Within each assay the LT_{50}s were higher at 10^5 than at 10^6 conidia/ml except for *Ae. vexans* at 10°C. In many of the assays, control mortalities were much too high to yield any meaningful LT_{50} estimates. This mostly occurred at the lowest temperatures with all species and at the highest temperature with *Ae. vexans* (Table 1).

The ST_{50}s followed much the same pattern as the LT_{50}s with 15 of 21 assays having no significant differences between the 2 estimated parameters at 10^6 conidia/ml. The ST_{50} estimates were generally one day less than the LT_{50}s.

There were significant differences in the mortality rates of the uninfected individuals at the different doses (Fig. 4). The control, 10^3 and 10^4 conidia/ml treatment uninfected mortalities were the same whereas significantly more individuals died without any signs of mycosis at 10^5 and 10^6 conidia/ml. This indicates that at the 2 lowest doses all mortality over and above control mortality can be attributed to mycosis. However, there were no significant differences in the proportions of infected to uninfected individuals between each dose.

DISCUSSION

Difficulties were encountered in the bioassay of *T. cylindrosporum* due to variability in the responses, the majority of which were not amenable to probit analysis. Similar results were obtained by Soares (1979,[3] 1982). In his studies

[3] Soares, G. G. Jr. 1979. A study of *Tolypocladium cylindrosporum* Gams, a new naturally occurring fungal pathogen of mosquitoes. Ph.D. Thesis, University of California, Berkeley. 177 pp.

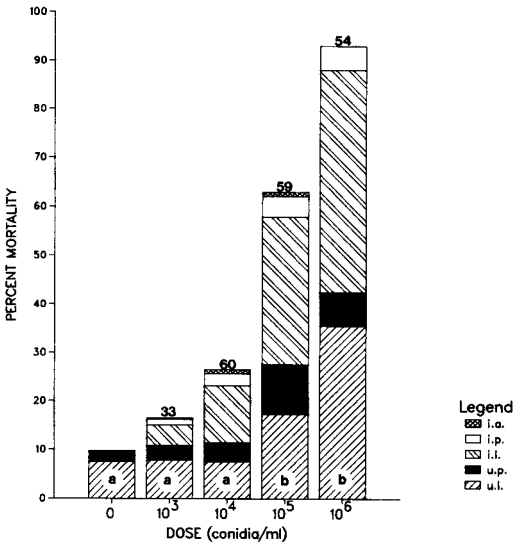

Fig. 4. Mortality of *Aedes aegypti* exposed to conidia of *Tolypocladium cylindrosporum* (TC3) at 20°C in relation to stage, infection diagnosis and dose. Data pooled from 6 assays of 60 larvae/dose/assay. Numbers above columns refer to mean proportion of total dead with diagnosed mycosis (%). There were no significant differences between these means ($F = 0.86$; $P = 0.48$). Letters within columns refer to significant differences between the mean mortalities of uninfected immatures ($F = 5.5$; $P = 0.003$) as determined by Scheffé's test at the 95% level of significance. i.a. = infected adults, i.p. = infected pupae, i.l. = infected larvae, u.p. = uninfected pupae, u.l. = uninfected larvae.

with *T. cylindrosporum* against *Ae. sierrensis* (Ludlow), 4 of 28 assays conducted were amenable to probit analysis; however, initial assays were hampered by poor yields and clumping of spores. Of 4 successful assays, 2 were heterogenous. Clearly, the bioassay method whereby mosquito larvae are continuously exposed to different concentrations of *T. cylindrosporum* is inadequate and needs major improvement. In the present assays that were amenable to probit analysis, the estimated median lethal concentrations and times were similar to those reported for *Ae. sierrensis* (Soares 1982, Soares and Pinnock 1984).

Sources of variation: Differences between batches of insects and pathogen as well as inaccuracies in the estimation of the dose are usually the principal sources of variation in bioassays (Bucher and Morse 1963, Burges and Thompson 1971). However, these are usually sources of variation that occur between assays. In the present bioassay of *T. cylindrosporum*, the major variability of response is occurring within assays as well as between assays. Since the assay method reported in this paper is similar to the one used successfully for *C. clavisporus*, the difficulties associated in the assay of *T. cylindrosporum* will be discussed relative to the assay of *C. clavisporus*.

As the assay system used is a closed one, larvae are continuously exposed to the inoculum. Therefore, the effective dose will vary according to the length of exposure as mosquitoes are reingesting the conidia again and again. Furthermore, since ingested conidia are still viable when excreted (Goettel 1987b[2]), ingestion of conidia is presumably not contributing to the nutrition of the mosquito. It is probable that the larvae at each higher conidial concentration are put under increasing stress as they ingest more and more non-nutritive matter (i.e., conidia) and thereby become more susceptible to mycosis. In his bioassays, Soares (1979,[3] 1982) used autoclaved conidia equivalent to the highest concentration in the assay as well as water with no conidia as controls, however he did not report any differences in the mortalities between these two. It is not known if mosquitoes are able to digest autoclaved conidia. Boiled conidia of *C. clavisporus* did not inflict significant mortalities in *Ae. aegypti* and *Anopheles hilli* Woodhill and Lee (Panter and Russell 1984, Sweeney 1983).

The bioassay is further complicated since each assay cup rapidly becomes a habitat of its own with a different microbial fauna. After a few days it was common to observe different turbidities among the assay containers. Such differences came about due to random contamination by microorganisms from the air and due to larval mortalities that subsequently affected the amount of food in each container. The abundance, nature and size of particulate matter in turn affect larval ingestion rates (Dadd 1970), thereby presumably affecting the amount of inoculum ingested. In addition the microbial fauna most likely has an effect on the viability of the conidia. Consequently, in this type of closed bioassay system, longer exposure times will tend to increase variability between replicates and doses.

Tolypocladium cylindrosporum conidia LT_{50}s are approximately 4 to 8 times greater than those of *C. clavisporus* (Sweeney 1983) while LC_{50}s are greater by factors of 10 to 100 (Cooper and Sweeney 1982). Sweeney (1976) found that his data were highly heterogenous when second instars were used; heterogeneity was reduced when first-instar larvae were used. The reduction of heterogeneity was probably a result of the more uniform physiological state of the younger larvae as well as their decreased median lethal time (Sweeney 1983). Subsequently, the assay method was further improved by using mortalities at 4 days rather than at 7 days (Cooper and Sweeney 1982). Dose-mortality responses of different instars to conidia of *T.*

cylindrosporum have not been well studied. Indications are that second-instar larvae are less susceptible to conidia than fourth-instars (Riba et al. 1986) while younger instars are more susceptible to blastoconidia (Pinnock et al. 1973[4]).

Possible improvements in assay technique: Since in *T. cylindrosporum* there is generally a direct relationship between dosage of conidia and mortality in the host population, it should be possible to develop an adequate bioassay by decreasing sources of variability. Possible ways to accomplish this would be to: 1) use very young larvae in order to decrease variability among them, 2) inoculate each assay container with a standard suspension of bacteria in an attempt to standardize the microbial fauna, 3) expose larvae to the inoculum for a limited time in order to standardize the dose as was done in the bioassay of *Nosema* (Jaronski 1979[5]) and 4) increase the number of doses so that the mortalities are evenly distributed around the LC_{50}s.

Host death and fungal colonization of hemocoel: At the lower doses, mortality rate of uninfected individuals was the same as for the control. At higher doses many more larvae died without fungal colonization of the hemocoel. It appears the increase in dosage of conidia has a detrimental effect on mosquito viability without fungal invasion of the hemocoel. Riba et al. (1986) also found a marked difference between percent mycosis and percent mortality in *Ae. aegypti* larvae exposed to *T. cylindrosporum* conidia.

The presence of ungerminated conidia in the guts of mosquitoes has been reported to be detrimental (Batschinsky 1927, Roberts 1970). In *C. clavisporus* death occurs within hours of ingestion of large quantities of conidia (Panter and Russell 1984). It was speculated that death was the result of release of toxins by germinating conidia. In the present study, however, deaths with no invasion of the hemocoel occurred throughout the exposure period. Since there were no differences in the proportions of dead immatures with and without mycosis between the different doses (Fig. 4), this phenomenon was occurring at the same rate at all doses.

In summary, it can generally be concluded that *T. cylindrosporum* is a relatively slow acting mosquito pathogen of low virulence which requires massive doses to elicit a response. Attempts to further evaluate *T. cylindrosporum* as a microbial control agent of mosquitoes are presently hampered by the lack of an adequate bioassay method. A better understanding of the host-pathogen relationships is needed.

[4] Initially described as *Beauveria tenella* but later confirmed as *T. cylindrosporum* (Soares et al. 1979).

[5] Jaronski, S. 1979. Role of the larval mosquito midgut in determining host susceptibility to *Nosema algerae* (Microsporida). Ph.D. Thesis, Cornell, Ithaca. 149 pp.

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