COMPARATIVE STUDIES OF *METARHIZIUM ANISOPLIAE* AND *TOLYPOCLADIUM CYLINDROSPORUM* AS PATHOGENS OF MOSQUITO LARVAE

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ABSTRACT. Mosquito fungal pathogens, *Metarhizium anisopliae* and *Tolypocladium cylindrosporum*, were compared with regard to virulence against the larvae of *Aedes aegypti, Anopheles stephensi* and *Culex pipiens. Culex pipiens* larvae were much more susceptible to *M. anisopliae* conidia than *An. stephensi* or *Ae. aegypti.* But *Ae. aegypti* and *Cx. pipiens* larvae were equally susceptible to *T. cylindrosporum* propagules which weakly attack *An. stephensi.* Using a high concentration conidial suspension (10^7 sp/ml) of *M. anisopliae* no. 139, *Ae. aegypti* larvae were killed immediately within 1.1 days, before intrahemocoelian invasion; but at lower concentrations (10^6 and 10^5 sp/ml), typical mycosis occurred. However, *T. cylindrosporum* no. 3 blastospores were much more pathogenic to *Ae. aegypti* larvae than conidia. Conidial suspension of 10^7 spores/ml killed 68% fourth-instar larvae, relative to the 96% invaded by blastospores under the same conditions. Presoaked conidia virulence appeared still intermediate between conidia and blastospores. At low temperatures, 15°C, virulence of *M. anisopliae* highly decreased, while at the same temperature, *T. cylindrosporum* blastospores were still virulent.

INTRODUCTION

Several species of fungi are currently being considered for use in the microbial control of mosquito larvae. These include *Lagenidium giganteum, Coelomomyces* spp., and deuteromycetes as *Culicinomyces clavisporus, Metarhizium anisopliae* or *Tolypocladium cylindrosporum.* A number of them seem to have promise on the basis of laboratory studies and preliminary field studies.

Metarhizium anisopliae is a good pathogen of mosquito larvae under laboratory conditions (Daoust and Roberts 1982, Balaraman et al. 1981). Several field tests have been carried out with this species. The results were sometimes promising (Roberts 1970, 1974, 1977) and other times disappointing (Mulla and Darwazeh 1980, Washino and Fetter-Lasko 1980). There is now an increased potential for *M. anisopliae* as a microbial agent for vector control in the light of recent formulation studies (Daoust et al. 1983; Daoust and Roberts 1983a, 1983b). *Tolypocladium cylindrosporum,* another hyphomycetous fungus, was discovered infecting mosquito larvae in 1978 (Soares et al. 1979). Although extensive field testing has not yet been carried out, laboratory studies have indicated that this species may have promise as a microbial control agent (Soares 1982, Weiser and Pillai 1981).

These studies were undertaken to compare the efficacy of *M. anisopliae* and *T. cylindrosporum* under controlled laboratory conditions against several mosquito species. One strain of each fungal species was used in these experiments. In each case, strain selection was based on an extensive screening of different strains, with the most virulent strain of each species being selected for these studies.

MATERIALS AND METHODS

FUNGAL STRAINS. The *M. anisopliae* strain selected was no. 139 (INRA, La Minière collection) isolated in 1981 from diapausing *Ostrinia nubilalis* larvae collected in central France. The *T. cylindrosporum* strain was no. 3 (same collection) isolated from *Aedes sierrensis* (Ludlow) larvae in northern California (Soares 1979)[2].

MOSQUITOES. The colony of *Culex pipiens* Linn. (Montpellier strain) was provided by the National Museum of Natural History, Paris; and the *Aedes aegypti* (Linn.) (Bora Bora strain) and *Anopheles stephensi* Liston (Kuwaiti strain) were provided by the Medical Entomology and Parasitology Department of ORSTOM, Bondy, France.

SPORE PRODUCTION. The conidia were produced in slant cultures of CM agar medium (KH_2PO_4, 0.36 g; $NaHPO_4$ 7 H_2O, 1.05 g; $MgSO_4$ 7 H_2O, 0.6 g; KCl, 1.0 g; NH_4NO_3, 0.7 g; glucose, 10g; yeast extract, 5.0 g; agar, 20 g; water, 1000 ml) incubated 15 days at 25°C. Blastospores were produced in shake cultures using an enriched medium (Adamek 1965).

BIOASSAY. Although the surface application of dry conidia is reported to be more efficacious for *M. anisopliae* (Roberts 1970, Daoust and Roberts 1982), we compared both fungi using aqueous suspensions since *T. cylindrosporum* has very hydrophilic conidia that cannot be

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[2] Soares, G. G. Jr. 1979. Study of *Tolypocladium cylindrosporum* Gams. a new naturally screening fungal pathogen of mosquitoes (with notes on *Beauveria bassiana* (Bals.) Vuill. Ph.D. dissertation, University of California, Berkeley.

induced to float for any length of time. Conidia were scraped from slant cultures, poured into distilled water, shaken with a Dangoumau machine at 700 pulses/min, and then passed through sieves (500 and 125 μm) to reduce clumping.

For each test 4 lots of 10 larvae in 50 ml of permuted[3] water in 100 ml plastic beakers were used for each treatment. Each bioassay also included 4 beakers containing 10 larvae each, which were not treated, and served as controls. All test containers were incubated at 25±2°C and checked daily for mortality. Larvae were fed daily using a ground rat chow diet (about 5 mg for 10 larvae). Dead larvae were removed and examined under the microscope in order to determine cause of death and check for hyphal growth. Assays were terminated at 5 days.

Time mortality data were analyzed using a computer program selecting a logit analysis option to calculate LT_{50}, with correction for control mortality by Abbott's formula.

RESULTS

RELATIVE VIRULENCE OF *M. ANISOPLIAE* AND *T. CYLINDROSPORUM* TO MOSQUITO LARVAE. At 25°C, conidia of *M. anisopliae* were more active against *Ae. aegypti* larvae than *T. cylindrosporum* conidia (Table 1). For example, among fourth-instar larvae exposed to *M. anisopliae* conidial suspensions of 10^6 spores/ml, 64% mortality was observed within 5 days; but using *T. cylindrosporum* conidia under the same conditions, only 31% mortality was noted. This conclusion was also confirmed on other mosquito species (Table 3).

[3] A resin-filtered water with enhanced natural Ca^{++} by new Na^{+} (G. Riba).

For *M. anisopliae*, second and third-instar larvae of *Ae. aegypti*, were a little more susceptible to infection than were fourth-instars. The LC_{50} values calculated at 5 days were 6.6×10^6 spores/ml for second instars, 3.7×10^5 spores/ml for third-instars, and 6.8×10^5 spores/ml for fourth-instars exposed to *M. anisopliae* conidia. On the contrary, second-instar larvae appeared a little more resistant than fourth-instars to conidia of *T. cylindrosporum*. LC_{50} values estimated 5 days after treatment with *T. cylindrosporum* conidia were 6.3×10^7 spores/ml for second-instars, and only 4.2×10^6 spores/ml for fourth instars.

The conidia of *M. anisopliae* were as pathogenic as the blastospores (Table 2). The immediate toxic effect seemed to be greater for conidia than for blastospores at the higher dosage. This can be seen from the LT_{50} which were 1.1 and 3.6 days, respectively. Several days postinoculation mortalities observed for both conidia and blastospores were very similar, as can be seen from the LT_{50} values.

This was not the case with *T. cylindrosporum*. For this species, blastospores were much more virulent than conidia. For example, at 10^5 spores/ml, the LT_{50} for blastospores was 4.1 days versus a calculated 26.6 days for conidia.

In order to explain this phenomenon we compared conidia and blastospores to conidia that were presoaked in liquid culture medium for 24 hr before being bioassayed. (At that time conidia were not still germinated.) At a low dose of 10^5 spores/ml we noted that the virulence of these presoaked conidia was intermediate between conidia and blastospores (Table 2). This indicated that a longer pregermination period for conidia may partly explain the differences in virulence observed between conidia and blastospores. Finally, *T. cylindrosporum* blastospores appeared much more pathogenic than *M. anisopliae* conidia against *Ae. aegypti* larvae (Tables 2 and 4).

Table 1. Comparative virulence of *Metarhizium anisopliae* (no. 139) and *Tolypocladium cylindrosporum* (no. 3) conidia to *Aedes aegypti* larvae at 25°C.[1]

		2nd instar				3rd instar				4th instar			
Fungus species and strains	dose sp/ml	Percent total mortality	LT_{50} (days)	Percent mycosis	LT_{50} by mycosis	Percent total mortality	LT_{50} (days)	Percent mycosis	LT_{50} by mycosis	Percent total mortality	LT_{50} (days)	Percent mycosis	LT_{50} by mycosis
Ma 139	10^7	88±5.4	1.0	27±16.4	—	100	1.0	50±8.2	4.9	95±1.8	1.1	64±24.6	2.9
	10^6	82.2+3.9	1.7	60±18.6	2.8	64±18.6	3.1	58±13.1	3	64±11.5	34.4	63±18.1	4.2
	10^5	59±7.8	4.1	51±14.9	4.5	48±9.4	—	43±22.4	—	22±15.6	—	15±8.9	—
	10^4	11±13.4	—	8±17.2	—	10±13.4	—	5±16.8	—	10±16.0	—	6±13.2	—
TC 3	10^7	40±5.9	—	30±9.1	—					68±8.4	3.6	66±14.1	—
	10^6	15±14.3	—	5±8.2	—					31±6.5	—	20±16.2	—
	10^5	5±4.9	—	5±4.1	—					25±21.7	—	20±21.4	—
	10^4	5±13.3	—	0	—					—	—	—	—

[1] Larvae exposed for 5 days; LT_{50} calculated after probit transformation; estimated LT_{50} values > 5.00 days not reported; calculated $LT_{50} < 1.0$ day noted 1.0 day because of daily control only; control mortalities (no spores applied) always ≤ 12.0% in control lots.

Table 2. Comparison at 25°C of different types of infectious propagules of *Metarhizium anisopliae* (no. 139) and *Tolypocladium cylindrosporum* (no. 3) against fourth instar larvae of *Aedes aegypti*[1].

Fungus species and strain	Dosage	Blastospores			Conidia			Pregerminated conidia		
		Percent total mortality	LT_{50}	LT_{90}	Percent total mortality	LT_{50}	LT_{90}	Percent total mortality	LT_{50}	LT_{90}
Ma 139	10^7	92.4±5.9	3.6	8.9	95±1.8	1.1	9	NT	NT	NT
	10^5	42.1±6.3	—	—	22±15.6	—	—	NT	NT	NT
TC 3	10^7	96±3.2	1.2	5.2	68±8.4	3.6	8.8	71.2±7.8	2.5	7.2
	10^5	65.2±13.3	4.1	—	25±21.7	—	—	31.2±16.0	—	—

[1] Total mortality is corrected for control mortality using Abbott's formula; LT_{50} and LT_{90} values calculated using logit analysis program; if mortalities < 50 or 90%, LT values not extrapolated; control lots mortalities (no spores applied) < 8.0%; presoaked conidia were incubated 24 hr in liquid culture medium before treatment.

SUSCEPTIBILITY OF DIFFERENT MOSQUITO SPECIES TO *M. ANISOPLIAE* AND *T. CYLINDROSPORUM*. The three mosquito species tested proved to be differentially susceptible to the two fungi. As reported by Daoust and Roberts (1982), *Cx. pipiens* was also the most susceptible species to *M. anisopliae* in our experiments. *Aedes aegypti* larvae were more resistant to this fungus than were *An. stephensi*. *Aedes aegypti* and *Cx. pipiens* were almost equally susceptible to *T. cylindrosporum*, while *An. stephensi* was substantially more resistant to this fungus (Table 3).

EFFECT OF TEMPERATURE ON *M. ANISOPLIAE* AND *T. CYLINDROSPORUM* PATHOGENICITY. The effectiveness of *M. anisopliae* and *T. cylindrosporum* was dependent on temperature. As temperature declined below 25°C the percent mortality declined and the LT_{50} increased. *Tolypocladium cylindrosporum* seemed to maintain somewhat higher activity at 15°C than did *M. anisopliae* with a less than two-fold increase in LT_{50} between 25°C and 15°C versus a greater than two-fold difference for *M. anisopliae* (Table 4).

DISCUSSION

These experiments have compared *M. anisopliae* and *T. cylindrosporum* against three important mosquito species. These data are particularly interesting since *M. anisopliae* generally invades through the siphon or integument (Al-Aidroos and Roberts 1978) while *T. cylindrosporum* spores can penetrate through the alimentary canal or the integument (Soares 1982).

Metarhizium anisopliae conidia and *T. cylindrosporum* blastospores appeared to be most promising against *Cx. pipiens* and *Ae. aegypti*, respectively. Our results were obtained not with randomly selected strains of each pathogen, but with strains that were selected from among 84 *M. anisopliae* and 11 *T. cylindrosporum* strains screened against *Ae. aegypti* (Riba et al. 1984, Soares et al. 1985). As such, the results were disappointing in that fairly high doses of propagules were required to achieve high mortalities in the host species tested. Only *M. anisopliae* conidia against *Cx. pipiens* and *T. cylindrosporum* blastospores against *Ae. aegypti* showed high mortality at the relatively high doses tested. However, Daoust and Roberts (1983a, 1983b) and Balaraman et al. (1981) reported better potencies of *M. anisopliae*. Also, Soares (1982) has already described the better efficacy of *T. cylindrosporum* blastospores than

Table 3. Comparative susceptibility of *Aedes aegypti*, *Anopheles stephensi* and *Culex pipiens* second instar larvae to *Metarhizium anisopliae* and *Tolypocladium cylindrosporum* conidia at 25°C[1].

Fungus species and strain	Doses (sp/ml)	*Aedes aegypti*		*Anopheles stephensi*		*Culex pipiens*	
		Percent Total mortality	LT_{50}	Percent total mortality	LT_{50}	Percent total mortality	LT_{50}
Ma 139	10^6	82.2±3.9	1.7	91±4.2	1.8	100	0.4
	10^5	59±7.8	4.1	76±15.9	2.3	92±3.4	0.8
TC 3	10^7	40±5.9	—	18±14.2	—	68±6.1	3.8
	10^6	15±4.3	—	11±13.9	—	32.5±9.4	—

[1] Larvae exposed for 5 days; calculated LT_{50} values > 5.0 days not reported; total mortality is corrected for control mortality using Abbott's formula; control mortalities (no spores applied) 8.0% with *Ae. aegypti*; 11.4% with *An. stephensi*; and 13.8% with *Cx. pipiens*.

Table 4. Influence of temperature on the infectivity of *Metarhizium anisopliae* conidia and *Tolypocladium cylindrosporum* blastospores to fourth-instar larvae of *Aedes aegypti*.[1]

Temperature	Ma 139 conidia		TC_3 blastospores	
	Percent mycosis	LT_{50} by mycosis	Percent mycosis	LT_{50} by mycosis
15°C	53±14.2	8.7	74±9.1	5.4
20°C	59±8.1	4.8	79±2.6	4.6
25°C	63±18.1	4.2	87±4.5	3.2

[1] Treatment at 10^6 sp/ml; incubated at 25°C; larvae exposed 9 days; control mortalities (no spores applied) 4.0% at 25°C; 8% at 25°C; 18% at 45°C. For this reason only percent mycosis were analyzed.

conidia to control mosquito larvae. Hall (1979) has described this in *Verticillium lecanii* on aphids.

Given that two different spore types are indicated for the two species of fungus tested in these experiments, one might consider the advantages and disadvantages of each (Keita 1982)[4]. Blastospores are readily produced in deep fermentation, while conidia are produced on aerial structures that require an aerobic environment on a solid or semi-solid media. Conidia can also be produced in surface culture on liquid media. Blastospores are generally considered to be less persistent than conidia (Ferron 1981). There is, however, very little data on the relative persistence of these two types of propagules in the aquatic environment and how different biotic and abiotic factors might influence persistence. Soares and Pinnock (1984) found that *T. cylindrosporum* conidia took considerably longer to germinate than blastospores. Furthermore, less than 1% of conidia placed in sterile tree hole water had germinated within one week at 25°C (Soares 1979). In these same experiments, blastospores were found to germinate readily in sterile distilled water and sterile tree hole water. Thus, it appears likely that conidia will generally persist better than blastospores in the aquatic environment.

Although *T. cylindrosporum* appeared to be more effective against *Ae. aegypti* than the other species tested, it was still less pathogenic to this host than to its natural host *Ae. sierrensis* (Ludlow). The LT_{50} was found to be about 14 hr for *Ae. sierrensis* exposed to 5×10^5 blastospores/ml (Soares and Pinnock 1984). With *Ae. aegypti*, the LT_{50} for blastospores was found to be 3.2 days at 1×10^6 blastospores/ml. Our data apparently showed an inverse relationship between larval mosquito instar and susceptibility to *Metarhizium*. This contrasts with the observations of Roberts (1970) and Balaraman et al. (1979) who applied the dry conidia on the surface water. We used an aqueous conidial suspension. During feeding, the larvae are able to fill their digestive tract with spores which will be disrupted, causing subsequent intoxication and death (Crisan 1971).

[4] Keita, A. 1982. Contribution à l'étude de la sensibilité des Culicidae (Diptères, Nematocères) à *Metarhizium anisopliae* (Metschnikoff) Sorokin et *Tolypocladium cylindrosporum* Gams. Thèse Doc. Ingénieur, Univ. Paris Sud, 66 p.

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