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LARVICIDAL ACTIVITY OF THE ENTOMOPATHOGENIC FUNGUS *TOLYPOCLADIUM CYLINDROSPORUM* (DEUTEROMYCOTINA: HYPHOMYCETES) ON THE MOSQUITO *AEDES TRISERIATUS* AND THE BLACK FLY *SIMULIUM VITTATUM* (DIPTERA: SIMULIIDAE)

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ABSTRACT. Laboratory bioassays evaluated the pathogenicity of the entomopathogenic fungus *Tolypocladium cylindrosporum* against *Aedes triseriatus*. All larval instars were found to be susceptible at temperatures from 18 to 25°C. Blastoconidia were more virulent than conidia. Mortality was proportional to exposure time in assays using blastoconidia. Blastoconidia and exposure time appear to be a more adequate method for bioassay of *T. cylindrosporum* virulence than the standard procedure of using conidia and continuous exposure. *Tolypocladium cylindrosporum* was also active against *Simulium vittatum* black fly larvae, but the mortality recorded was much lower than with mosquito larvae.

INTRODUCTION

The fungal class Deuteromycotina contains more than 40 identified entomopathogens including *Tolypocladium cylindrosporum* Gams, a known pathogen of mosquitoes (Saunders et al. 1988). In the Culicidae, numerous larval species and one adult species, *Aedes sierrensis* (Ludlow), have been recorded as hosts (Soares 1982). But it is not known if *T. cylindrosporum* has any activity against *Aedes triseriatus* Say, a vector of LaCrosse virus (Watts et al. 1975), or against Simuliidae. The activity against mosquito larvae over a broad range of temperatures (Soares and Pinnock 1984, Riba et al. 1986, Goettel 1987) suggested that *T. cylindrosporum* could be a potential candidate for control of mosquito and black fly larvae in aquatic habitats in temperate areas. The fungus produces 2 types of vegetative spores, conidia (on solid media) and blastoconidia (in liquid media), of which the blastoconidia are more virulent (Soares 1982, Soares and Pinnock 1984, Riba et al. 1986).

Most laboratory bioassays are performed by continuously exposing mosquito larvae to the fungal spores. The field situation is quite different. Particulate larvicides, like a spore suspension, used over stagnant, semistagnant, or running water, will sink and/or move away from the site of application. Larvae are exposed for a limited time to the spores. Thus it is important to examine contact time.

This paper reports the larvicidal activity of *T. cylindrosporum* conidia and blastoconidia against the larvae of *Ae. triseriatus* and the black fly *Simulium vittatum* Zetterstedt.

MATERIALS AND METHODS

Larvae: A strain of *Ae. triseriatus* collected near Trois-Rivières (Québec, Canada) was reared in the laboratory according to Munstermann and Wasmuth (1985). A photoperiod of 18:6 (L:D) was used for rearing the larvae and performing the bioassays. *Simulium vittatum* larvae were collected from the Réserve Faunique du Saint-Maurice, located 110 km north of Trois-Rivières, and maintained at 4°C in a water-circulating system where current was generated along removable plastic plates (larval substrate) using compressed air (Lacoursière and Boisvert 1987).

Fungal inoculum: Tolypocladium cylindrosporum (University of Alberta Microfungus Collection, Edmonton, Canada: #4561) was maintained at 20°C by serial transfer on Pablum® cereal agar (Pablum, 50 g; agar, 9 g; distilled water, 500 ml). Conidia were obtained by washing 2-wk-old fungal cultures growing on the surface of Pablum agar with 80–100 ml of distilled water. Blastoconidia were produced in 250 ml Sabouraud-dextrose broth (Difco, 0382-17-9). One-liter flasks were inoculated with pieces (4 × 1 cm) of agar covered with actively growing mycelium, and incubated at 20°C on a rotary shaker (125 rpm). After 3–4 days, the blastoconidia and mycelium were separated by filtering the whole culture through a screen cloth (mesh opening 0.25 mm). The filtrate was centrifuged at 700 × *g* for 30 min. The spores were collected and centrifuged–washed twice. The absence of mycelia in the spore suspensions was confirmed by microscopic observations.

Conidia and blastoconidia concentrations were determined with a Petroff-Hausser cell counter,

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Table 1. Mortality of *Aedes triseriatus* larval instars after continuous exposure to *Tolypocladium cylindrosporum* conidia.[1]

Concentration (spores/ml)	Cumulative percentage mortality per instar[2]			
	I	II	III	IV[3]
1×10^7	71	47	50	90
1×10^6	6	42	0	35
1×10^5	13	42	4	18
1×10^4	13	25	11	5

[1] Each assay was performed in triplicate using 25 larvae. Cumulative mortality after 9 days exposure at 20°C.
[2] Corrected for control using Abbott's formula.
[3] The concentration used was 4.2 time higher for the 4th-instar larvae.

Table 2. Mortality of *Aedes triseriatus* 2nd-instar larvae after continuous exposure to *Tolypocladium cylindrosporum* conidia at different temperatures.[1]

Concentration (spores/ml)	Cumulative percentage mortality[2]		
	18°C	20°C	25°C
1×10^7	46	45	55
1×10^6	18	38	15
1×10^5	12	38	15
1×10^4	29	22	4

[1] Each assay was performed in triplicate using 25 2nd-instar larvae and was stopped after 9 days exposure at 20°C.
[2] Corrected for control using Abbott's formula.

and the suspensions were kept at −20°C. Before each bioassay, the viability of the spores was determined by spreading 1 ml of the suspension onto the surface of potato-dextrose agar (Goettel 1987) (BBL, 11550) and incubating at 20°C. After 24–48 h, the percentage of viable spores was counted for 5 random microscopic fields (400×) and the dilution was adjusted to the desired concentration. The conidia (solid medium) were considered viable if they swelled to a barbell or peanut shape or if they formed a germ tube (Goettel 1987, 1988a). The blastoconidia (liquid medium) were considered viable if they produced a germ tube. Viability of both spore types was always greater than 85%.

Bioassays: The virulence of *T. cylindrosporum* was assayed by varying larval instar of *Ae. triseriatus,* temperature, type of spore (conidia and blastoconidia), and exposure time. The mosquito analyses were done by exposing batches of 25 larvae to 10-fold dilution of spores ranging from 10^4 to 10^7 spores/ml. The larvae were placed in plastic cups containing 100 ml of distilled water, maintained at 20°C, and fed every 2 days with 0.2 ml of a liver powder suspension (6 g/100 ml). The water level was kept at 100 ml by adding distilled water. Triplicates were made for each spore concentration used, with duplicates for controls: one with autoclaved spores, the other without any spores. Daily mortality was recorded, and the dead larvae were removed. The bioassays were stopped when, depending on the bioassay, pupation or adult emergence occurred.

Studies of temperature effects at 18, 20, and 25°C used 2nd-instar larvae of *Ae. triseriatus* exposed to different spore concentrations. To study the effect of exposure time, blastoconidia were used at the concentration corresponding to the optimum mortality obtained against 2nd-instar larvae at 20°C. The larvae were exposed to spores for 30 min, 1 h, 2 h, 6 h, and continuously, followed by filtration through a fishnet. The larvae were washed with distilled water and put in a new plastic cup with 100 ml of fresh distilled water and 0.2 ml of the liver suspension.

Because of the results obtained with *Ae. triseriatus* larvae, *T. cylindrosporum* blastoconidia were tested against black fly larvae in the laboratory. The assays were performed with the system described by Lacoursière and Charpentier (1988), using batches of 50 3rd-instar field-collected *S. vittatum* larvae. The larvae were exposed continuously to 4 concentrations of blastoconidia (10^3, 10^4, 10^5, or 10^6 spores/ml). One control consisted of 10^6 autoclaved spores/ml, and the other had no spores. Triplicates were used for each concentration and control. The experimental temperature and photoperiod were 10°C and 18:6 L:D. The mortality was recorded daily, and the dead larvae were removed. The assays were stopped following pupation. All the mortalities were corrected for controls using Abbott's formula (Abbott 1925).

RESULTS

After 9 days of exposure to various concentrations of conidia, all larval instars were found to be susceptible to *T. cylindrosporum* at the selected conidial concentrations (Table 1). The highest average cumulative mortality observed with the highest spore concentration was 90% at the 4th instar. But the concentration was 4.2 times higher for the 4th instar larvae. As expected, the lower concentrations gave lower and variable mortalities. Second instar larvae were selected for the other bioassays because this larval instar shows greater mortality at all concentrations of conidia below 10^7 spores/ml. Second-instar larvae were used to assess the effect of temperature

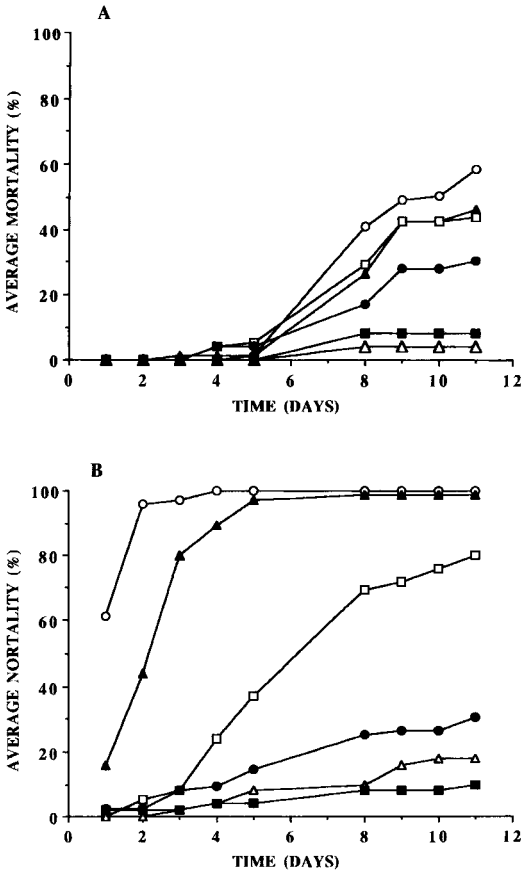

Fig. 1. Cumulative percent mortality of 2nd-instar larvae of *Aedes triseriatus* continuously exposed to various concentrations of *Tolypocladium cylindrosporum* conidia (A) and blastoconidia (B) at 20°C. Each concentration was tested in triplicate using 25 larvae. ○: 1×10^7 spores/ml; ▲: 1×10^6 spores/ml; □: 1×10^5 spores/ml; ●: 1×10^4 spores/ml; △: control without spores; ■: control with 1×10^7 autoclaved spores/ml.

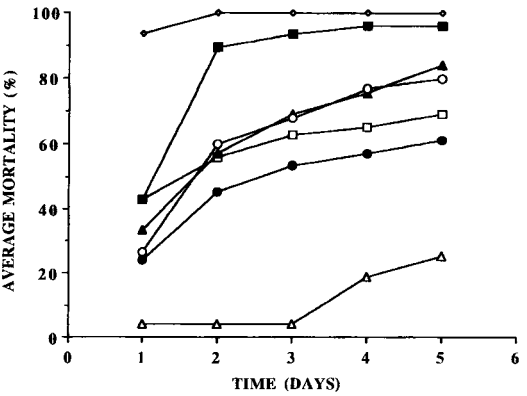

Fig. 2. Cumulative percent mortality of 2nd-instar larvae of *Aedes triseriatus* during different exposure times to *Tolypocladium cylindrosporum* blastoconidia (1×10^6 spores/ml) at 20°C. Assays were done in triplicate using 25 larvae per test. ●: 15 min; □: 30 min; ▲: 1 h; ○: 2 h; ■: 6 h; ◆: continuous exposure; △: control without spores.

on larval mortality (Table 2). Mortality varied with spore concentration, with a maximum of 55% at 25°C with 10^7 spores/ml. At the median concentrations (10^5–10^6 spores/ml), mortality was highest at 20°C.

Based on these results, a temperature of 20°C was selected to compare the activity of the 2 types of spores. Mortality appeared 5–6 days after continuous exposure to each concentration (Fig. 1A) of conidia and varied between 30 and 60% after 11 days. However, blastoconidia elicited faster and higher larval mortality (Fig. 1B). After 5 days, 100% mortality was reached at the concentrations of 10^6 and 10^7 blastoconidia/ml.

Tolypocladium cylindrosporum was also effective when larvae were exposed to blastoconidia for various periods of time (Fig. 2). The highest mortality was obtained for the continuous exposure period (100% mortality at day 2) and declined with the decrease in the length of time that the larvae were exposed to blastoconidia. The minimum value recorded at day 3 was 53% for 15 min of contact time, but mortality continued to increase during the following days. The assays were stopped at day 6 because control mortality was too high.

Simulium vittatum larvae continuously exposed to blastoconidia started to die 9 days after the beginning of the experiment. Only the highest concentration (10^6 spores/ml) resulted in mortality greater than 60% if we consider that at day 18 the control mortality was 20% (Fig. 3).

DISCUSSION

Tolypocladium cylindrosporum was found to kill *Ae. triseriatus* larvae, a new host to be recorded with other susceptible *Aedes* species. All instars were susceptible from 18 to 25°C, with the blastoconidia being more virulent than the conidia. Comparable results for both types of spores of *T. cylindrosporum* have been reported for other mosquito species (Soares 1982, Soares and Pinnock 1984, Riba et al. 1986). The difference between blastoconidial and conidial activity was correlated with faster germination of the blastoconidia (Soares and Pinnock 1984, Riba et al. 1986). The speed at which *T. cylindrosporum* blastospores killed mosquito larvae suggested that a metabolic toxin might be involved

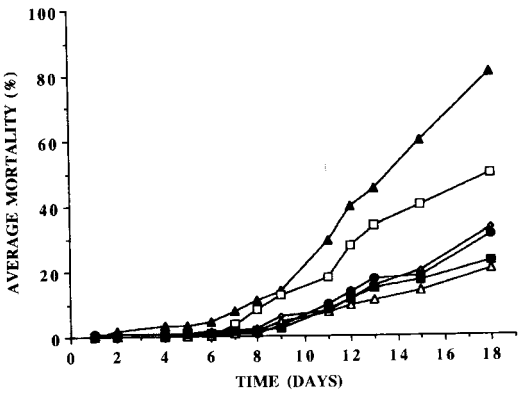

Fig. 3. Cumulative percent mortality of *Simulium vittatum* larvae continuously exposed to various concentrations of *Tolypocladium cylindrosporum* blastoconidia at 10°C. Fifty larvae were exposed at each concentration. ▲: 1×10^6 spores/ml; □: 1×10^5 spores/ml; ●: 1×10^4 spores/ml; ◆: 1×10^3 spores/ml; △: control without spores; ■: control with 1×10^6 autoclaved spores/ml.

(Soares 1982). A toxin called "tolypin", larvicidal for both mosquito and black fly larvae, has been extracted from 3–8-wk agar slant cultures of *T. cylindrosporum* by Weiser and Matha (1988).

The response of larval instars to *T. cylindrosporum* has not been well studied. Second-instar *Aedes aegypti* (Linn.) were found to be more resistant to conidia than the 4th instars (Riba et al. 1986). In our study with *Ae. triseriatus,* this was observed only at the highest concentration of conidia. At lower concentrations, the 2nd-instar larvae were more sensitive than all other instars. The variation in results between larval instar can be explained in part by the feeding behavior of *Ae. triseriatus.* According to Merritt et al. (1978) and Merritt (1987), most particles ingested by *Ae. triseriatus* are smaller than 50 μm, with the particle size ingested increasing with larval instar, 1st and 2nd instars prefer a size smaller than 2 μm, the 3rd instars a size between 2 and 10 μm, and 4th instars prefer a size between 10 and 25 μm. In a natural environment *Ae. triseriatus* not only filters the food particles in the water column but also can grind or graze the microflora (bacteria, fungi, etc.) (Fish and Carpenter 1982). This feeding behavior was also observed in the bioassay cups. Even if the common infection site of *T. cylindrosporum* is the integument, the conidia can pass through the alimentary tract, and be excreted and remain viable (Soares 1982; Goettel 1988a, 1988b). As *T. cylindrosporum* conidia measure 3.5–6.8 × 1.2–1.9 μm, they are ingested by filtration principally by the 3rd and 4th instars, whereas the 1st and 2nd instars will grind the conidia. If the conidia contain a toxin, it is released by the grinding 1st- and 2nd-instar larvae (by crushing the conidia) causing, as we have observed, more mortality than in the 3rd and 4th instar filtering larvae. The difference in mortality between the 1st and 2nd instars can be explained by an increase in feeding of the older instar.

A proportional relationship was found between mortality and exposure time to blastoconidia of *T. cylindrosporum* (Fig. 2). This is the first time that this relationship is reported for *T. cylindrosporum.* We recorded good mortality with all the exposure times, even at a 15-min contact time.

According to Goettel (1987) continuous exposure of larvae to various concentrations of conidia is not an ideal bioassay system for fungal virulence. He identified factors to explain variability between replicates and doses. The effective dose can vary according to the length of exposure, as mosquitoes are reingesting conidia that are still viable when excreted; the variability between replicates could be caused by the microbial fauna developed in the assay cup after few days. These sources of variability might be corrected by using blastoconidia and a contact time strategy. Using short contact times will reduce the reingestion of spores. Because blastoconidia are more virulent than conidia, bioassays are shorter and, therefore, the development of the microbial fauna and other variables are reduced.

We report here the first instance of virulence of *T. cylindrosporum* blastoconidia to black fly larvae. Black fly larvae are much less susceptible to infection than mosquitoes. This difference could possibly be explained by the temperature of exposure; 10°C for *S. vittatum,* compared to 20°C for *Ae. triseriatus.* Lower germination percentage for blastoconidia when the temperature decreases from 20 to 10°C has been related to a decrease in mortality (Soares and Pinnock 1984).

Culicinomyces clavisporus Couch, Romney and Rao is the only other deutermoycete that has been tested against black fly larvae (Knight 1980, Gaugler and Jaronski 1983, Sweeney and Roberts 1983). This fungus, like *T. cylindrosporum,* was less active against black flies than mosquitoes (Gaugler and Jaronski 1983, Sweeney and Roberts 1983). Sweeney and Roberts (1983) concluded that *C. clavisporus* did not merit serious consideration against simuliid larvae. It is difficult to compare pathogenicity/virulence of the 2 fungus species because experimental conditions were different. We believe that the mortality observed in this study, and the results using differ-

ent contact times on mosquito larvae, are interesting enough that *T. cylindrosporum* merits more consideration for use as a biocontrol agent for black flies.

To evaluate this fungus as a biological control agent, bioassays using blastoconidia and variable exposure times are an alternative to using conidia and continuous exposure. The results with different contact times and temperatures indicates the possibility that the fungus could be used as a larvicide against mosquitoes and black fly larvae in temperate climates.

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