

## PATHOGENICITY, HOST RANGE AND TEMPERATURE TOLERANCE OF *CRYPTICOLA CLAVULIFERA* (OOMYCETES: LAGENIDIALES) IN THE LABORATORY

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**ABSTRACT.** The Oomycete fungus *Crypticola clavulifera* was pathogenic for laboratory-reared and field-collected larval Diptera, including the mosquitoes *Aedes aegypti*, *Ae. notoscriptus*, *Anopheles farauti* No. 1 and 3, *Culex annulirostris*, *Cx. quinquefasciatus* and the chironomid midge *Chironomus tepperi*. Zoospores were produced in water held at 15, 20 and 25°C and were pathogenic for *Ae. aegypti* larvae, but at 30 and 35°C zoospores were not produced.

Oomycete fungi from the Lagenidiales have potential as biocontrol agents for medically important Diptera, especially mosquitoes. The most promising of these is *Lagenidium giganteum* Couch, which has been the subject of considerable research in the last decade (Lacey and Lacey 1990). Another entomogenous fungus from this group, *Crypticola clavulifera* Humber, Frances and Sweeney, was isolated in 1985 from the larvae of *Forcipomyia marksae* Tokunaga (Diptera: Ceratopogonidae) collected from the leaf axils of a taro in a rainforest at Millaa Millaa, northern Queensland, Australia (Frances et al. 1989). The biology of *C. clavulifera* is similar to *L. giganteum*, where motile zoospores actively seek susceptible hosts, but there are differences between the 2 in the production and liberation of zoospores from infected larvae. Zoospores of *C. clavulifera* develop wholly within spherical sporangia and are discharged through a short papular extension, whereas the cytoplasmic contents of *L. giganteum* sporangia are extruded into an evanescent vesicle where zoospores differentiate and are released. In this note preliminary laboratory studies on the pathogenicity, temperature tolerance and host range of *C. clavulifera* are reported.

Initial experiments were conducted to observe the pathogenicity of *C. clavulifera* for mosquitoes and other dipterous larvae. The fungus was grown on PYG4S (0.125% peptone, 0.125% yeast extract, 0.3% glucose, 1.2% agar plus 0.00625% each of cholesterol, lanosterol, ergosterol and cholestan- $\beta$ -ol and 0.01% lethicin) plates for 4 days, and then inoculated into duplicate flasks containing 50 ml of "Z" medium, using the method of Domnas et al. (1982). The flasks were placed onto a rotary shaker at 200 rpm for 6 days at 25°C. In one infection experiment, 3 ml of culture and medium were added to a tray containing 300 ml of distilled water. Approximately  $10^5$  zoospores/ml were observed in the test tray after 18 h when 200 2nd instar

*Aedes aegypti* (Linn.) larvae were added. Twenty-four hours later, several dead larvae had multiple encystment of *C. clavulifera* zoospores on their cuticles. After 48 h, 100% mortality was observed in the challenged larvae. For another experiment, 30 ml of the original "Z" medium was poured through a coarse filter to concentrate the hyphal material, which was then placed into a tray containing 300 ml of distilled water. After 18 h, zoospore concentration was estimated with an improved Neubauer hemocytometer to be  $10^5$  zoospores/ml. These zoospores were further diluted to  $10^4$  zoospores/ml in 4 trays containing 270 ml of distilled water. Second instar larvae were added to test and fungus-free trays as follows: 70 *Culex annulirostris* Skuse, 70 *Cx. quinquefasciatus* Say, 70 *Ae. aegypti* and 40 *Anopheles farauti* No. 3, with one species per tray. All larvae were fed with 0.2 ml of autoclaved yeast suspension (5% w/v) per day, and survivors counted after 3 days. The mortalities in test trays were 31.4% for *Cx. annulirostris*, 42.9% for *Cx. quinquefasciatus*, 84.3% for *Ae. aegypti* and 5.0% for *An. farauti*. In all control trays mortality was less than 5%. Dead larvae from test trays of each species were observed with internal growth of *C. clavulifera* sporangia.

*Crypticola clavulifera* was grown on PYG4S for 4 days at 25°C, then inoculated into 3 flasks of PYG3S broth (same ingredients as PYG4S, without agar and ergosterol) and 3 flasks of "Z" medium. The flasks were incubated on a rotary shaker at 200 rpm at 25°C for 6 days. Trays containing 200 ml of distilled water were inoculated with 5 ml of each culture. After 20 h, when zoospores of *C. clavulifera* were produced, 50 2nd instar *Ae. aegypti* larvae were added to each test tray and to fungus-free controls. Larvae were fed on days 0, 1, 2, 3 and 4 with autoclaved yeast, and survival was recorded on day 6. Mortality of larvae added to trays with inoculum from PYG3S was between 96–100% compared with 10–27% for "Z" medium. The reduced mortality of the latter may be due to a

lack of exogenous sterols and nutrients in the "Z" medium. The legal restrictions associated with obtaining hemp seed used in "Z" medium and the low mortalities in this experiment prompted the use of PYG3S for routine production of zoospores.

Two experiments were conducted on the effect of water temperature on the development and pathogenicity of *C. clavulifera* for *Ae. aegypti* larvae. In the first, fungal inoculum was grown in PYG3S broths for 7 days. Four test and 2 control trays containing 300 ml of distilled water were placed in incubators at 15, 20, 25, 30 and 35°C. Each test tray was inoculated with 5 ml of *C. clavulifera* hyphal growth. After 21 h, 50 *Ae. aegypti* larvae were added to each tray, and larvae were fed 0.3 ml of autoclaved yeast on days 0, 1 and 3. Surviving larvae were scored on day 6. The mean corrected mortality in test trays held at 15, 20, 25, 30 and 35°C was 99, 99.5, 99.5, 0 and 0%, respectively. Microscopic examination of the inoculum in trays held at 30°C and 35°C showed the fungus was killed at these temperatures, and zoospores were not produced.

In the second temperature trial, test trays were inoculated with 5 ml of fungal hyphae and media and placed in incubators set at 15, 20, 25 and 30°C. Three successive groups of 2nd instar *Ae. aegypti* larvae (50 per tray) were added to each test tray 18, 42 and 66 h post-inoculation and removed after 24 h exposure. A fourth group of larvae was also added 90 h post-inoculation to trays held at 15°C. After removal, each group of larvae was placed into trays containing 300 ml of distilled water and returned to the same temperature conditions. Larvae were fed autoclaved yeast on day 0, 1 and 3, and survivors counted on day 6 (Table 1). Zoospore production was not detected at 30°C, and the mortality of larvae was very low. At 20°C and 25°C, zoospores remained viable for up to 66 h post-inoculation, while at 15°C, the onset of zoospore production was delayed; but zoospores remained motile for over 90 hours. The larvae that died in these assays were examined for the production of *C. clavulifera* sporangia and zoospores. At all temperatures, the percentage of dead lar-

Table 2. Percentage of dead *Aedes aegypti* larvae producing *Crypticcola clavulifera* zoospores at different temperatures.

Temperature (°C)	Hours of exposure to <i>C. clavulifera</i>			
	18-42	42-66	66-90	90-114
15	55.6	21.9	50	92.9
20	98.4	100	100	—
25	90	95	84.4	—
30	0	0	—	—

vae with fungal growth and zoospore production was high. At 20°C and 25°C, greater than 84% of all dead larvae were infected and producing zoospores (Table 2).

In an earlier study the ceratopogonids *Forcipomyia marksae* and *Dasyhelea* sp., and *Ae. aegypti* were reported as susceptible hosts of *C. clavulifera* (Frances et al. 1989). During the current study the host range of the fungus has been expanded to include the larvae of several species of Culicidae, Chironomidae and Ceratopogonidae. The mosquitoes *An. farauti* No. 1 and 3, *Cx. quinquefasciatus* and *Cx. annulirostris*, all from laboratory colonies maintained at Ingleburn, and the local container breeding *Aedes notoscriptus* (Skuse), were infected. Larvae of the midge *Chironomus tepperi* (Skuse), from Yanco, NSW, and an unidentified ceratopogonid were also infected in laboratory challenges. *Aedes kochi* (Dönitz) larvae from Millaa Millaa, the original site of fungus discovery, were not susceptible in these experiments.

The results indicate that *C. clavulifera* is pathogenic for a number of medically important Diptera. These experiments were conducted within 12 months of the isolation of the fungus, and in challenges after this time the fungus had a reduced ability to produce zoospores and to infect mosquito larvae. Lord and Roberts (1986) reported the prolonged maintenance of *L. giganteum* on medium without added sterols was detrimental to vigor. They indicated this was a gradual process and that routine maintenance on sterol free media should be avoided. Although

Table 1. Effect of temperature on the pathogenicity of *Crypticcola clavulifera* for 2nd instar *Aedes aegypti* larvae.

Temperature (°C)	Mean mortality (% ± SE) of <i>Ae. aegypti</i> * exposed for 24-h periods post-inoculation			
	18-42 h	42-66 h	66-90 h	90-114 h
15	32.0 ± 2.4	65.0 ± 7.7	94.5 ± 5.5	100
20	84.5 ± 9.7	100	100	—
25	84.0 ± 9.3	100	96.0 ± 2.3	—
30	3.0 ± 1.0	3.0 ± 1.3	—	—

\* Mean of 4 replicates.

this isolate of *C. clavulifera* was routinely grown on PYG4S media containing sterols, the fungus has lost vigor. Future studies with this isolate may require passage of the fungus through mosquito larvae.

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