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PILOT SCALE PRODUCTION AND APPLICATION IN WILDLIFE PONDS OF *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES)

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ABSTRACT. *Lagenidium giganteum,* a facultative parasite of mosquito larvae, has recently been registered by the U.S. Environmental Protection Agency for operational mosquito control. We report here the first pilot scale production of the mycelium formulation. Scale-up from 10 to 650 liters was accomplished by a proportionate increase of medium components and volume of water. Foaming of the culture medium had not been encountered previously, but was a serious problem in pilot scale production due to the very rapid growth of a large volume of *L. giganteum.* Addition of an antifoaming agent did not adversely affect growth, but reduced the ability of the fungus to sporulate. Despite what was effectively a 100-fold reduction in the desired application rate due to reduced sporulation, *L. giganteum* infected sentinel mosquito larvae and reduced field populations for more than 2 months following application.

Lagenidium giganteum (Oomycetes: Lagenidiales), a facultative fungal parasite of mosquito larvae, is the only biological control agent approaching operational use in mosquito control. The infective stages of the fungus are biflagellate, motile zoospores that selectively adhere to larval cuticle. Infection is initiated by mechanical and enzymatic activity of the encysted zoospore, allowing entry into the larva, with larval death occurring within 24–72 h. Upon maturation of the mycelium, the fungus can reproduce either sexually or asexually. Asexual reproduction amplifies infection, with a new round of zoospore release occurring every 24–72 h, depending upon the mosquito host and environmental conditions. Sexual reproduction culminates in the production of oospores, which are dormant propagules capable of surviving prolonged desiccation, environmental extremes, and mechanical abrasion. These spores allow multiyear persistence of *L. giganteum* in some habitats (Fetter-Lasko and Washino 1983, Kerwin and Washino 1986).

Three formulations of *L. giganteum,* consisting of various combinations of the sexual and asexual stages, have been registered with the U.S. Environmental Protection Agency (USEPA Registration Nos. 56984-1, 56984-2, and 56984-3), with the Department of Health Services, State of California, acting as registrant. This biological control agent can be produced with inexpensive medium components using standard stirred tank fermentation (Kerwin and Washino 1986, 1987), and, with some precautions, applied by ground or air with techniques and equipment currently used by mosquito control agencies (Kerwin and Washino 1988). This report summarizes pilot scale fermentation production of the fungus, and demonstrates that even suboptimal applications can result in larval mortality in a wildlife habitat.

A preliminary test was conducted 2 wk before the field application to insure that the shear forces generated by the circulation pump used by the aircraft to deliver the material to the spray booms would not damage *L. giganteum* mycelia. Mycelia grown in 2 10-liter fermentation batches was filtered, concentrated in distilled water, and mailed to the Sacramento-Yolo Mosquito Abatement District, Sacramento, California. This material was diluted in water in the hopper of the plane that was to be used for the field application. The pilot then flew the plane under conditions similar to those to be used during application. At 5-min intervals, for a total of 20 min, the pilot landed and a small sample was removed from the hopper of the aircraft, with no significant loss in viability observed even after 20 min.

The *L. giganteum* mycelium formulation (USEPA Registration No. 56984-2, American Type Culture Collection Accession No. 52675) was used for these field trials. Inoculum for large-scale fermentation was grown in 10 liters of medium in 14-liter tanks in a 3-tank New Brunswick Scientific Co. Labroferm FS-314 unit using slight modifications of the yeast extract-based medium described by Kerwin and Washino (1988). The inoculum was grown for 60 h, and 18 liters was then aseptically collected in a sterile 20-liter carboy. This material was transported to Bainbridge Island, WA, and used to inoculate 650 liters of the medium described above in a 1,000-liter-capacity custom-built fermentor.

The agitation rate was 200 rpm, which, due to the length of the propellors and geometry of the tank, was equivalent to *ca.* 350 rpm in our standard 10-liter fermentation tanks. In the 1,000-liter fermentor, the fungus was aerated initially at a rate of 3,000 cc/min, but this rate has to be gradually cut back to *ca.* 2,000 cc/min *ca.* 15 h

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after beginning the fermentation, and then finally to 1,500 cc/min after 30 h because the fungus was growing so rapidly that it was foaming out the top of the fermentor between the agitator gaskets. This happened despite the automatic injection of a liter of antifoam (Mazur, DF 6,000 K) over a period of 12 h as the fungus initiated rapid growth that caused excessive foaming.

Harvesting of the cells was initiated 60 h after inoculation. The fungus was concentrated and resuspended in distilled water in 20-liter carboys after about a 15-fold concentration. The following day the carboys were surrounded with bags of ice, and transported to Davis, CA, where they were stored at 15°C until application.

Sufficient material for treatment of the test site at an approximate rate of 4.5×10^{10} cells/ha was transported to an airstrip on Conaway Ranch, adjacent to the field sites to be treated. A sample of this mycelia was kept in the laboratory, and germination and virulence to laboratory-reared *Culex pipiens* Linn. assessed in both distilled water and in samples of water drawn from each of 5 plots treated with the fungus on the Conaway Ranch. This was done using a concentration of fungus about 100× that of the field application to facilitate quantitative analysis of sporulation.

When mycelium from the material used for the field applications was diluted in distilled water, all 36 sentinel *Cx. pipiens* larvae added to the pan were dead and infected by *L. giganteum* in 15 h. Larval infection was confirmed by microscopic examination. There was no larval infection in field-site water after 15 h, but after 3 days infection was 100% in all of the water samples from the field sites; however, when the percentage of cells that released zoospores was assessed after 72 h, 61% of those in distilled water had released zoospores, but less than 1% of the cells suspended in water from the field sites had released zoospores. This compares with the usual levels of greater than 98% of cells releasing zoospores in distilled water and greater than 90% germination in water from comparable field sites when the fungus was grown in the same media in liquid shake culture or 10-liter fermentors. These results suggested that the effective field application rate of the fungus was less than $\frac{1}{100}$ of that required for immediate high levels of mosquito control. Subsequent laboratory evaluations using liquid shake cultures confirmed that the antifoam agent delayed zoosporogenesis and reduced the percentage of reproductively competent cells.

For field applications, the concentrated fungus was diluted in sufficient water (previously loaded into the hopper of the plane) to allow an application rate of 10 liters/ha (*ca.* 1 gallon/acre). A Schweizer AgCat was used to apply the mycelium using a Transland spray system with an air-driven pump. Air speed was 95–100 mph and the swath width was 10.5 m (40 ft.). The uniformity and density of mycelial application was checked in the following manner. Fungus sprayed from the plane was collected from 3 passes in a 20-cm-diam plastic bucket holding 200 ml water. This material was returned to the laboratory, concentrated by filtration through paper filter, and the collected cells examined microscopically. Approximately 40 cells were examined, and all appeared undamaged by the application process. These cells were subsequently resuspended in distilled water, and 30 colony-reared *Cx. pipiens* larvae added to the pan. After 3 days there was 100% infection of these larvae.

Applications of the fungus were made in 3 separate fields on the Conaway Ranch Conservancy located several miles northwest of Sacramento, California. The most heavily monitored field, 51 ha (127 acres) in size, was subdivided into 12 plots. Plots 1 through 5 were treated with *L. giganteum*; plot 6 was a buffer; plots 7 through 9 were treated with the Vectobac® formulation of *Bacillus thuringiensis* var. *israelensis* at an effective rate of 1.25 liters/ha (1 pint/acre); and plots 10 through 12 served as controls. Two other fields, 55 ha and 65 ha, were treated with fungus alone.

Pretreatment dipping was done in all fields 24–48 h prior to application of the control agents, using 10 dips per plot. Aquatic light traps were placed in selected locations to monitor aquatic invertebrates, and 2 sentinel buckets, each containing 50 3rd-instar laboratory-reared *Culex tarsalis* Coq. larvae, were placed in each plot and in the 2 outlying fields. Nontarget sampling of invertebrates and monitoring of fungal activity with sentinel larvae was repeated approximately every 2 wk for 2 months. Sampling of indigenous larvae by personnel from the Sacramento-Yolo Mosquito Abatement District was done weekly from August 7 through October 6 using standard 0.47-liter (1-pint) dippers. Ten dips were taken in a transect across all plots of a given treatment. In past studies, we returned all larvae that were collected from dip samples to the laboratory, where they were held for several days to monitor infection. Because the mosquito abatement personnel were monitoring on an operational basis and had limited time for this study, an alternate sampling strategy was used. Larvae sampled with the dippers were pooled as the number of 1st- plus 2nd-instar, and 3rd- plus 4th-instar larvae. This allowed rough estimates of egg oviposition over the previous 2–4 days, and survival of younger instars to older larvae.

The laboratory evaluations of sporulation described above suggested that initial fungal activ-

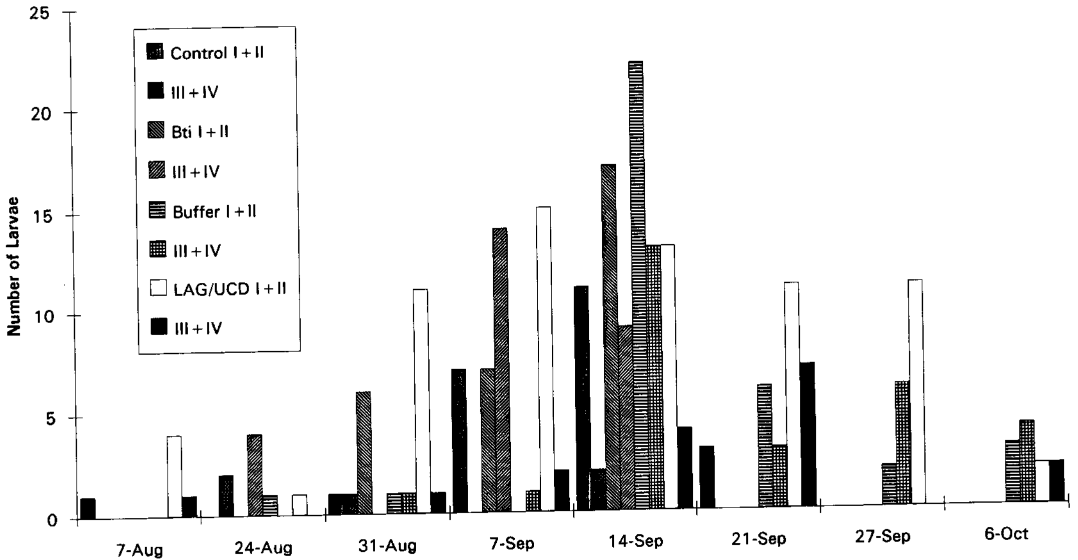

Fig. 1. Number of larvae collected in 10 dips from control plots, buffer plot, and plots treated with either *B.t.i.* or *Lagenidium giganteum*. Data are reported as number of (1st + 2nd) instars and number of (3rd + 4th) instars. August 7 corresponds to day 22 postapplication.

ity in the experimental plots would be minimal. This was confirmed during the first 2 wk of sentinel monitoring when no infection occurred during the initial monitoring period, and only one *Lagenidium* plot had sentinel infection (11% infection of sentinel larvae) 2 wk postapplication. It was decided to continue monitoring the treated fields to see if the initial very low levels of infection would be amplified as the larval breeding season continued.

This strategy was successful beginning 4 wk after application, when *L. giganteum* infections were documented in 3 of the 5 test plots treated with the fungus. Sentinel larvae were also infected in a second *Lagenidium*-treated plot, designated the levee field, and in one of the sites treated with *B.t.i.*, which was located 4 plots away from the nearest site treated with the fungus. The fungus was possibly transferred to the *B.t.i.* check by waterfowl, either by adherence to feathers and feet, or by passage of fungus-infected larvae through the gut following ingestion. This phenomenon was observed again during the last sampling period, which was 2 months after fungal application, when sentinels were infected with *L. giganteum* in 2 of the *B.t.i.* plots.

Percent sentinel infection levels in the 5 adjacent plots treated with *L. giganteum* were: 5 days posttreatment—0%; 16 days—2 ± 5%; 29 days—47 ± 38%; 43 days—5 ± 10%; 63 days—13 ± 9% (mean ± 1 SD, $n = 5$).

Dip sampling of the 12 plots suggested that *L. giganteum* reduced indigenous late instar (3rd and 4th instars) larval populations beginning 3 wk after applications (August 7 sampling date), and continuing with varying degrees of success for the following 3 wk (Fig. 1). *Culex tarsalis* was the dominant species during the first part of the sampling period, and *Anopheles freeborni* Aitken was abundant during the second half. Reduction of the abundance of late larval instars by the fungus was especially pronounced during September. It should be stressed that the larval numbers are an instantaneous estimate of abundance, and did not take into account larvae that were infected but still alive, or would be infected before pupation. Larval abundance figures for the *L. giganteum* plots, therefore, are an underestimation of the true impact of the fungus on the mosquito population.

A very rough estimate of oviposition vs. survival was obtained by separating the abundance of the 2 younger instars from the 2 older stages. There were up to 8 times more young (1st- and 2nd-instar) larvae compared to old instars in the plots treated with *L. giganteum* during 4 of the 8 sampling periods. Given the limitations of this sampling technique, we suggest that recycling of the fungus was probably impacting the larval populations.

Aquatic light trap collections and microscopic examination of nontarget invertebrates, which included Coleoptera, Diptera, Hemiptera, Mollusca, and Annelida showed no adverse effects on these organisms following fungal application.

There is substantial precedent for fermenting

filamentous fungi such as *Penicillium* and *Aspergillus* spp. for commercial production of enzymes and pharmaceuticals, and *Beauveria bassiana* and *Metarhizium anisopliae* for insect control. This is also true for various strains of bacteria including the toxin-producing *Bacillus thuringiensis,* which have been grown commercially in large-scale fermentors for several decades. By comparison, oomyceteous fungi have rarely been cultivated in batches larger than one liter, and there is no information on industrial-scale production of this group of organisms. It is fortunate, therefore, that media developed for liquid shake and small-scale fermentation production of *L. giganteum* (Jaronski et al. 1983, Kerwin et al. 1986) appear to be suitable for larger scale production. The complication in production of this fungus for mosquito control is that mycelia can readily be produced that are incapable of either asexual or sexual reproduction. Motile zoospores must be produced by one of these 2 modes of reproduction for larvae to be infected, so mycelial production *per se* cannot be used to assess the usefulness of a given set of culture protocols.

Current fermentation yields for the asexual stage of *L. giganteum* are comparable to those for the microbial insecticide *B.t.i.* This stage, then, would appear to be ideal for large-scale industrial production; however, the problems with the asexual stage include its short shelf life, the need to keep the mycelium completely hydrated, its susceptibility to being overwhelmed by contaminating microorganisms following formulation, lack of stability under extremes of temperature, and special handling required to keep the formulated product from becoming anaerobic (Domnas et al. 1977, 1982; Jaronski et al. 1983; Lord and Roberts 1985; Su et al. 1986).

There have been attempts to mitigate these problems by manipulating storage conditions of agar cultures (Su et al. 1986) or encapsulating mycelium in calcium alginate (Axtell and Guzman 1987). Although these attempts have had some degree of success, mycelial stability is still limited to less than *ca.* 3 months. More importantly, the cost and effort involved with these formulations are prohibitive in the current mosquito control market.

We were successful in our first attempt at pilot scale production of *L. giganteum,* which is not expected except for the very simplest fermentations. The one complication we encountered was the adverse effects of the antifoam agent used. An alternative to using antifoaming agents is to increase the fermentor tank head pressure, and it is possible that this strategy alone will solve the foaming problem during future fermentations.

The oospore or sexual stage, unlike the asexual stage, is a thick-walled spore, resistant to abrasion and desiccation, which can be stored for at least 7 years and still remain viable in either the laboratory or the field (Fetter-Lasko and Washino 1983, Brey and Remaudiere 1985, Kerwin and Washino 1988). Low fermentation yields, which are currently 2 orders of magnitude below what is necessary for commercial viability, are the major impediment to use of oospores for operational mosquito control. Optimization of oospore yields is a major focus of our current research efforts.

The major advantage of using this agent rather than other control methods has been amply demonstrated in this paper. If *B.t.i.* or any chemical agent had been applied at less than 1% of their recommended rates, mosquito control would have been negligible, and the breeding site would have had to be immediately retreated. By treating with a microbial agent capable of recycling and amplifying the applied material, mistakes in application due to operator error or equipment calibration can be compensated for by this versatile and adaptable fungus.

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