GROUND AND AERIAL APPLICATION OF THE SEXUAL AND ASEXUAL STAGES OF *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES) FOR MOSQUITO CONTROL[1, 2]

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ABSTRACT. Fermentor-grown cultures of the sexual and asexual stages of *Lagenidium giganteum* were applied in rice fields in the Central Valley near Sacramento, CA. Both ground and aerial applications of the asexual stage resulted in high levels of immediate control of sentinel *Culex tarsalis* and indigenous *Cx. tarsalis* and *Anopheles freeborni* larvae and provided some degree of control throughout the four-month mosquito breeding season. Oospores which were desiccated in the field following application provided consistently high larval infection levels after reflooding of the fields. Advantages of using the sexual stage of *L. giganteum* for field larval control are presented.

INTRODUCTION

The facultative parasite *Lagenidium giganteum* Couch (Oomycetes: Lagenidiales) has shown sufficient efficacy in small scale field trials to warrant consideration as an alternative to chemical control of larvae (Fetter-Lasko and Washino 1983, Jaronski and Axtell 1983, Kerwin and Washino 1983a, 1985). Development of in vitro production methods of the sexual stage of the fungus on solid media (Kerwin and Washino 1983b) and in liquid shake and fermentation culture (Kerwin et al. 1986) has enhanced the potential for large scale commercial use of *L. giganteum*. The asexual stage is easily grown in liquid shake culture using modifications of methods devised by Domnas and colleagues (Domnas et al. 1977, 1982) and results in high larval mortality within several days after its introduction into a suitable environment; however, this stage in the life cycle of the parasite is fragile and must remain hydrated to maintain its viability. Oospores are a dormant stage of *L. giganteum* capable of withstanding desiccation, temperature extremes and mechanical abrasion (Fetter-Lasko and Washino 1983, Kerwin and Washino 1983b). These spores remain viable when stored dry at room temperature for at least 7 years (Kerwin et al. 1986).

The following report describes field evaluation of fermentor-grown cultures of the sexual and asexual stages of *L. giganteum* in rice fields in the Sacramento Valley, CA. Results from ground applications and larger scale aerial treatments are reported and recommendations made for future research which will enhance the operational use of the fungus for mosquito control.

MATERIALS AND METHODS

SOURCE AND MAINTENANCE OF THE FUNGUS. The California strain (ATCC 52675) of *L. giganteum* was used in these investigations (Kerwin and Washino 1983b). The isolate was periodically reisolated from oospore-infected *Culex tarsalis* Coquillett and maintained in liquid culture on one of the following media: 1) 1.5 g/liter autolyzed yeast extract (Ardamine pH, Desmo Chemical Corporation, Elmsford, NY), 0.5 g/liter glucose, 0.5 g/liter triolein, 0.05 g/liter soybean lecithin, 0.025 g/liter cholesterol, 2 mM CA^{2+}, 1 mM Mg^{2+}, pH 6.5. 2) 1.5 g/liter Ardamine pH, 1.0 g/liter glucose, 1 ml/liter corn oil, 0.05 g/liter cholesterol, 2 mM Ca^{2+}, 1 mM $Mg2^{2+}$, pH 6.5. Transfers of stock cultures were made using zoospore suspensions 12–18 hr after induction of zoosporogenesis by dilution of ca. 10 ml of 7- to 14-day old cultures in sterile distilled water. Stock cultures were grown in cotton-stoppered 500 ml Erlenmeyer flasks containing 100 ml of media. Cultures were shaken at 110–120 rpm on a rotary shaker under ambient temperature (23 ± 3° C) and light conditions for a minimum of 5 days. All lipid supplements were obtained from Sigma Chemical Company.

For inoculation of fermentation tanks the fungus was grown for 24–48 hr in 1 liter of medium in 2800 ml fernbach flasks containing 2.0 g/liter Ardamine pH, 2.0 g/liter glucose, 1 ml/liter corn oil, 0.05 g/liter cholesterol, 2 mM Ca^{2+}, pH 6.5. Each fermentation tank was inoculated with 500 to 1000 ml media.

[1] The process of in vitro oospore culture described in this paper is subject to pending patent legislation.

[2] *Lagenidium giganteum* is not currently registered by the EPA for use. Studies carried out on the Colusa Wildlife Refuge were performed under special use permit #514 issued by the United States Fish and Wildlife Service, United States Department of the Interior. The studies in Sutter County were performed under Experimental Use permit #52300-EUP-1 issued by the United States Environmental Protection Agency. The treated crop was destroyed at the end of the growing season. Safety tests using *L. giganteum* have been initiated in conjunction with Dr. J. Shadduck, University of Illinois at Urbana-Champaign, to facilitate its registration.

FERMENTATION CULTURE OF *L. GIGANTEUM*. For field trials of the asexual age, the fungus was grown in 12 liters of media in either a New Brunswick Scientific (NBS) Microferm MF 214 fermentor or a NBS MA 114 fermentor (14-liter tanks, 12 liter working volume). The medium used for the asexual stage evaluations was 15 g Ardamine pH, 10 g glucose, 2 g dried egg yolk, 4 g $CaCl_2.2H_20$, 2 g $MgCl_2.6H_20$, 0.8 g cholesterol, 3 ml cod liver oil (Möller's), 7 ml wheat germ oil, pH 6.5, in 12 liters distilled water. Operating conditions were 28°C, 100 rpm and 2.0–2.5 × 10^3 cc/min air.

For the ground applications the cultures were grown for 5 days following inoculation of each tank with 500 ml of stock culture. This was sufficient time to allow initial maturation of oospores based on extensive investigations of this process (Kerwin et al. 1986). For the aerial trials, media for asexual cultures were inoculated with 1 liter of stock culture and grown for 60 hr under these conditions. Although oosporogensis was initiated after 60 hr under these conditions, further development of oospores was interrupted by collection of the mycelium at this time, and no viable oospores were present in these cultures.

To obtain sufficient quantities of the asexual stage for aerial application, three separate series of fermentations had to be carried out in each of the three 14-liter tanks available for use. After 60 hr of fermentation, mycelium was filtered from the growth medium onto nylon mesh (50 micron) and resuspended in several liters of distilled water in plastic carboys. Ampicillin was added to limit bacterial growth and the cultures were stored at 12–15°C until initiation of the aerial trials. The oldest cultures were stored for 7 days prior to application.

A variety of media were used to supply oospores for the field trials. Oospores were stockpiled over a period of 18 mo as gradual modifications were made in culture methodology (Kerwin et al. 1986). The basal growth media in all of the cultures consisted of Ardamine pH, glucose, cholesterol, vegetable oils (corn, wheat germ or linseed) and/or cod liver oil, various additional carbon and nitrogen sources (peptone, liver extracts, egg byproducts) and millimolar amounts of calcium and magnesium.

Oospores and mycelium were collected by filtration on nylon mesh 7 to 14 days after inoculation of the fermentation tanks. The number of viable oospores was determined using a hemocytometer and previously described morphological characteristics (Kerwin and Washino 1983b) and the mycelium plus oospores was filtered and dried at room temperature. The mycelium/oospore mixture was subsequently washed 4–6X by blending in distilled water at high speed in an Osterizer® blender followed by filtration of the suspension. This process reduced the percentage of aborted oospores frequently encountered following rehydration of dried oospore preparations (Kerwin et al. 1986). The washed oospores were dried once more and stored at room temperature in plastic containers until field application.

GROUND APPLICATION OF THE SEXUAL AND ASEXUAL STAGES. Initial field tests involved application of the fungus in rice fields maintained at the Colusa Wildlife Refuge near Colusa, CA, for feeding migratory birds. *Lagenidium giganteum* was applied in an aqueous suspension using a compressed air backpack sprayer with its nozzle removed. Five 20 × 20 m plots, separated by a minimum of 20 m, were treated between June 15 and July 15, 1985, with 5-day old cultures at a rate of 1.5 liters culture medium/10 m^2. This material contained ca. 5.5 × 10^7 viable oospores/1.5 liters medium. One plot was treated as above but an additional 10^8 dehydrated oospores were sprayed uniformly throughout the plot. Six 2 × 2 m plots, separated by 10 m, were treated with 6-month old oospores at rates of ca. 10^5, 2 × 10^5 or 4 × 10^5 oospores/plot.

Sentinel cages (Case and Washino 1979) containing 25 second or third instar laboratory-reared *Cx. tarsalis* larvae were used to monitor activity of the fungus. Five cages were used in each of the 20 m^2 plots and 1 cage in each 2 m^2 plots. Sentinel mosquitoes were placed in plots on the day of application and periodically thereafter until the fields were drained in early September. Sentinel cages remained in the field for 3 to 4 days. Larvae were then collected and returned to the laboratory to evaluate percentage infection. Dead larvae were examined immediately under a dissecting scope to confirm infection by *L. giganteum*. Living larvae were held 3 days following collection from the field to assess latent parasitism. Some sentinel infection may have been caused by recycling of the fungus within the sentinel cages since 3 days is sufficient for completion of the asexual cycle of the fungus. Over 80% of the infected larvae were dead when collected from the sentinel cages, suggesting they were parasitized by inoculum already present in the field. This relatively long exposure time gives a good indication of the control potential of a biological agent which is active over a prolonged period of time.

All plots were monitored for fungal activity prior to treatment using sentinel mosquitoes, with no infection recorded. Indigenous populations of larvae were periodically monitored using standard 1 pint (0.47 liter) dippers, 50

dips/20 m^2 plot. Water samples were taken at the time of fungal application for analysis of pertinent characteristics and a record was kept of maximum/minimum water temperatures.

AERIAL APPLICATION OF *L. GIGANTEUM*. The fungus was applied by air from a seed hopper using corn cobs as an inert carrier. The asexual stage (mycelium) was concentrated into a slurry and mixed with 300 pounds (136.4 kg) of corn cobs previously dampened with water. *Lagenidium giganteum* grows in small clumps of oval cells similar to yeasts, and mixes much more uniformly than a typical filamentous fungus. The wetting step decreased desiccation of the fragile mycelium, which was applied at a rate equivalent to 1.1 liter medium/10 m^2. The sexual stage was dampened just prior to mixing with 300 pounds of cobs and applied at ca. 10^{10} oospores/hectare using an Ag Cat biplane.

Plots were staked in a commercial rice field in the Sutter Bypass south of Yuba City, CA. Each plot was 50 × 200 m (1 hectare) and separated from adjacent plots by a minimum of 200 m. The swath width of the application was set so that 3 passes over each plot provided overlapping coverage of the entire area. Material was applied in the early afternoon at a rate such that most of the cob/fungus mixture was delivered after passing over the time of application.

One sexual, one asexual and one control plot were evaluated. Each plot was monitored for fungal activity twice a week using sentinel mosquitoes and dipping of the indigenous larval population. Fifteen sentinel cages, each with 25 second or third instar laboratory-reared *Cx. tarsalis*, were placed in a 70 m linear transect along the center of each of the treated plots. There was no fungal activity in either the treated or control plots prior to initiation of the trials as monitored by sentinel mosquitoes. Sentinel infection was evaluated after alternating 3- and 4-day periods in the field using the protocol previously outlined. Four laboratory personnel sampled the indigenous larval population along the sentinel cage transect with each sampler taking 100 dips using a 1 pint dipper over a total distance of 175 m. Dipping, collection of old sentinel mosquitoes and placement of new sentinel larvae was done on the same day. The control transect was dipped using the protocol described above to assess the larval population change in the absence of *L. giganteum* application. Water samples were taken the day of fungal application for screening of pertinent characteristics and a record was kept of maximum/minimum water temperatures.

RESULTS

GROUND APPLICATION OF THE ASEXUAL STAGE. Initial evaluation of the efficacy of fermentor-grown mycelium for mosquito control using the asexual stage of *L. giganteum* revealed several unexpected results. In the absence of a significant larval population, the first 2 plots treated showed high sentinel mortality up to 10 days after application (Fig. 1). This gradual release of zoospores is in contrast to the behavior of the asexual stage grown in liquid shake culture in which less than 50% sentinel infection is apparent after 6 days (Kerwin et al. 1986). Recycling of the fungus is negligible at very low indigenous larval densities (Kerwin et al. 1986) and by 17 days after treatment there was no detectable fungal infection of sentinel larvae (Fig. 1). The plot which was treated with dried oospores as well as the 5-day old material did not show any added fungal activity.

Three weeks after treatment of the first 2 plots the rice check in which they were located was dry because of a malfunction of the water pump used to flood the field. These plots remained dry for ca. 8 days, after which they were reflooded to the proper water level. Within 10 days of reflooding there was significant infection of sentinel larvae in the oospore-supplemented plot, and fungal activity persisted at high levels throughout the remainder of the test (Fig. 1). When the second plot was again checked for the presence of *L. giganteum* zoospore release 2 weeks after reflooding, sentinel infection was significant (Fig. 1). This initial activity was due entirely to activation of oospores in the field since the mycelium could not have survived the 8-day period of desiccation. The oospore-supplemented plot consistently produced greater sentinel mortality than this latter plot (Fig. 1).

Two of the three plots treated with the asexual stage during a second series of trials also exhibited prolonged zoospore release following application (Fig. 1). All 3 of these plots exhibited sporadic fungal activity throughout the remainder of the monitoring period at levels characteristic of recycling of the fungus (Fig. 2). There was sufficient indigenous larval density during this time for recycling to proceed in contrast to the situation early in the breeding season (Fig. 1).

The temperature range in the test plots during the course of these trials was 19–32°C, which bracketed the operational range of the fungus. Total nitrogen and 11 other ions in the water at the time of application were well within the concentration range tolerated by *L. giganteum*.

GROUND APPLICATION OF OOSPORES. Six smaller plots were treated with oospores which had been dried and stored in the laboratory for 6 months. Germination of oospores in this field

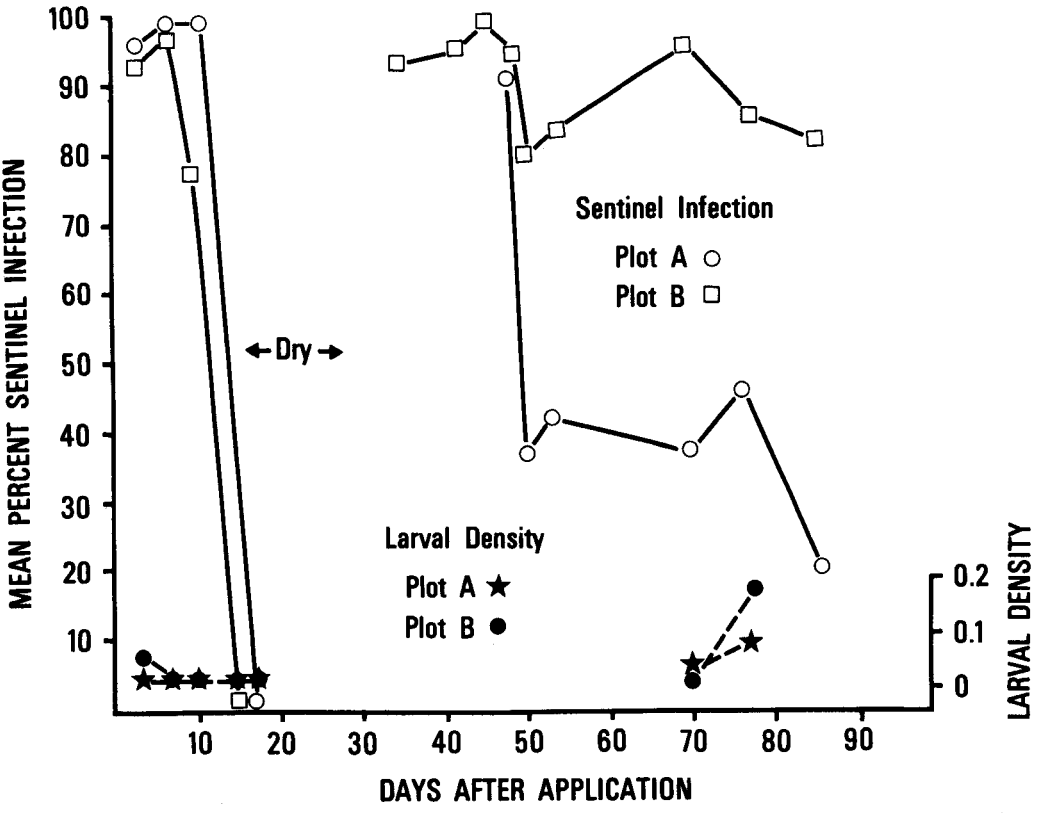

Fig. 1. Infection of *Culex tarsalis* sentinel larvae and density of indigenous larvae (*Culex tarsalis* and *Anopheles freeborni*): Ground applications of *L. giganteum*, plot A treated with asexual stage with mature, nonactivated oospores and plot B treated with asexual stage with activated (desiccated) and nonactivated oospores. Data referring to percent sentinel infection is based on larvae from 5 sentinel cages. Data referring to larval density is the average of 50 dip samples.

test was typical of that observed in the laboratory (Kerwin et al. 1986), i.e., a low level of germination occurring over several months. Activation of the dormant oospores was sporadic and unpredictable, with sentinel infection ranging from 0 to 100% (Fig. 3). It is assumed that only a small fraction of the oospores germinate at any one time. Under laboratory conditions less than ca. 20% of a given lot of oospores have germinated after 70 days of rehydration using our present maturation and activation techniques. Larval density in the vicinity of these smaller plots was less than 0.05 larva/dip throughout the course of these tests; therefore, it is assumed that most of the observed sentinel infection was due to direct infection by activated oospores.

AERIAL APPLICATION OF *L. GIGANTEUM*. Aerial application of *L. giganteum* in two 1-hectare plots was performed in early August 1985 to demonstrate the feasibility of larger scale field evaluations of this fungus and to delineate any obvious technical problems in preparation for more intensive evaluations planned for 1986. The 2 formulations of the sexual and asexual stages were prepared just prior to their application. Although the asexual material was applied at a theoretical rate of 1.1 liter/10 m^2, mortality of the fungus could have been as high as 40% of the inoculum due to storage of some of the mycelium for up to 1 week before application, abrasion from mixing with corn cobs, desiccation and failure to reach the rice field water. This estimate is based on microscopic evaluation of a small sample of the formulated material brought back to the laboratory after field application.

Despite these complications, the asexual application successfully reduced the indigenous larval population and resulted in gradually decreasing (99+% initially to 38% at the end of the trial) sentinel larval infection (Fig. 4). Oospore germination was very sporadic as monitored by sentinel larval infection (Fig. 4) and had no apparent effect on the indigenous larval population until the last day of sampling. It is

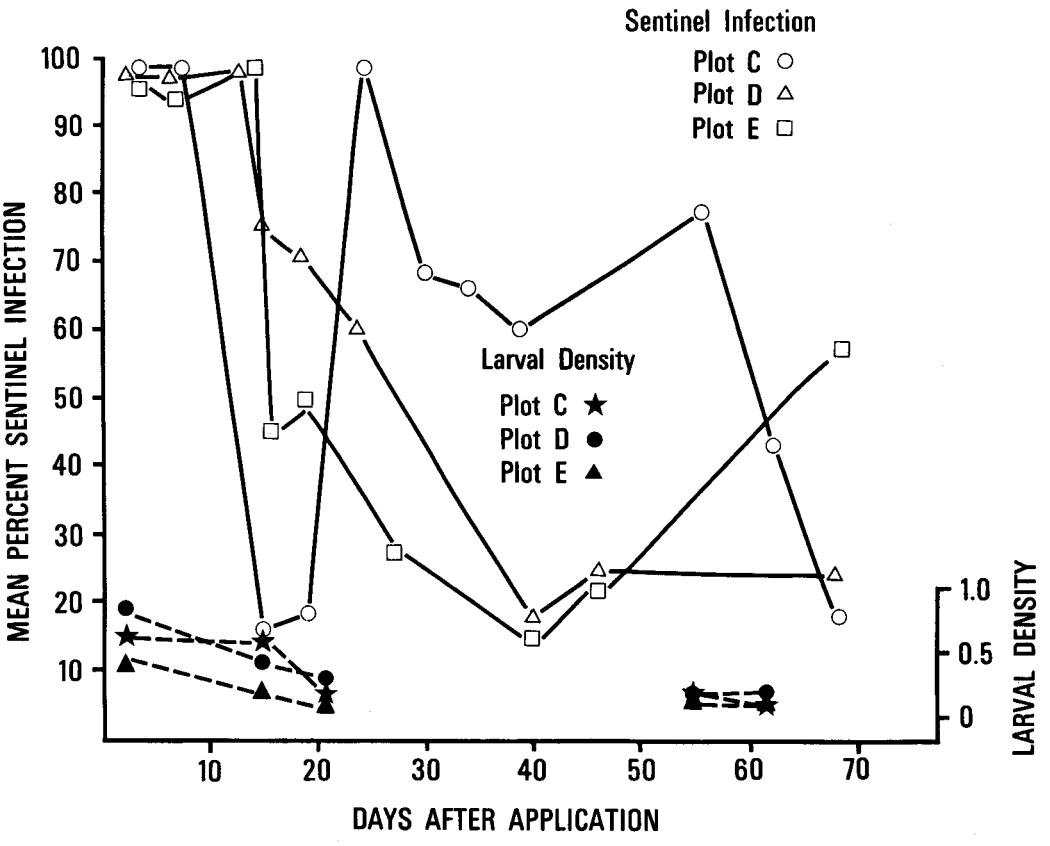

Fig. 2 Infection of *Culex tarsalis* sentinel larvae and density of indigenous larvae (*Culex tarsalis* and *Anopheles freeborni*): Ground applications of *L. giganteum*, Plots C, D, and E, each treated with the asexual stage plus mature nonactivated oospores. Data referring to percent sentinel infection is based on larvae from 5 sentinel cages. Data referring to larval density is the average of 50 dip samples.

not clear from the sentinel infection data that this observed reduction was due to oospore-induced mortality.

DISCUSSION

Previous field tests with *L. giganteum* have used in vivo cultures of the fungus (Umphlett and Huang 1972, McCray et al. 1973, Fetter-Lasko and Washino 1983), or in vitro asexual cultures grown on agar (Jaronski and Axtell 1983) or in liquid shake culture (Kerwin and Washino 1983a, 1985). Although these techniques are amenable to small scale trials, especially in habitats where the potential for epizootics exists, larger scale tests require the use of alternate culture techniques such as fermentation. A second possibility is to rely on small scale production of oospores and stockpile this resistant stage over several months or years. This paper reports the first use of fermentation culture to produce both the sexual and asexual stages of *L. giganteum* on a scale sufficient for aerial application.

Use of the asexual stage is relatively easy for field control efforts up to several hectares or tens of hectares if larger fermentation tanks, e.g., 130 liter working volume or more, are available. Growth requirements for the culture of mycelium capable of asexual zoosporogenesis are less strict than those for sexual reproduction. Also, the induction of zoosporogenesis, which is necessary for mosquito infection, can be routinely accomplished over a fairly predictable time period by dilution of the mycelium in water. Routine use of this relatively fragile stage on a very large scale is limited since it requires constant hydration and handling.

With the development of techniques for the in vitro production of oospores (Kerwin and Washino 1983b, Kerwin et al. 1986), the potential for commercial production and operational use of *L. giganteum* for mosquito control has been enhanced. Current yields from

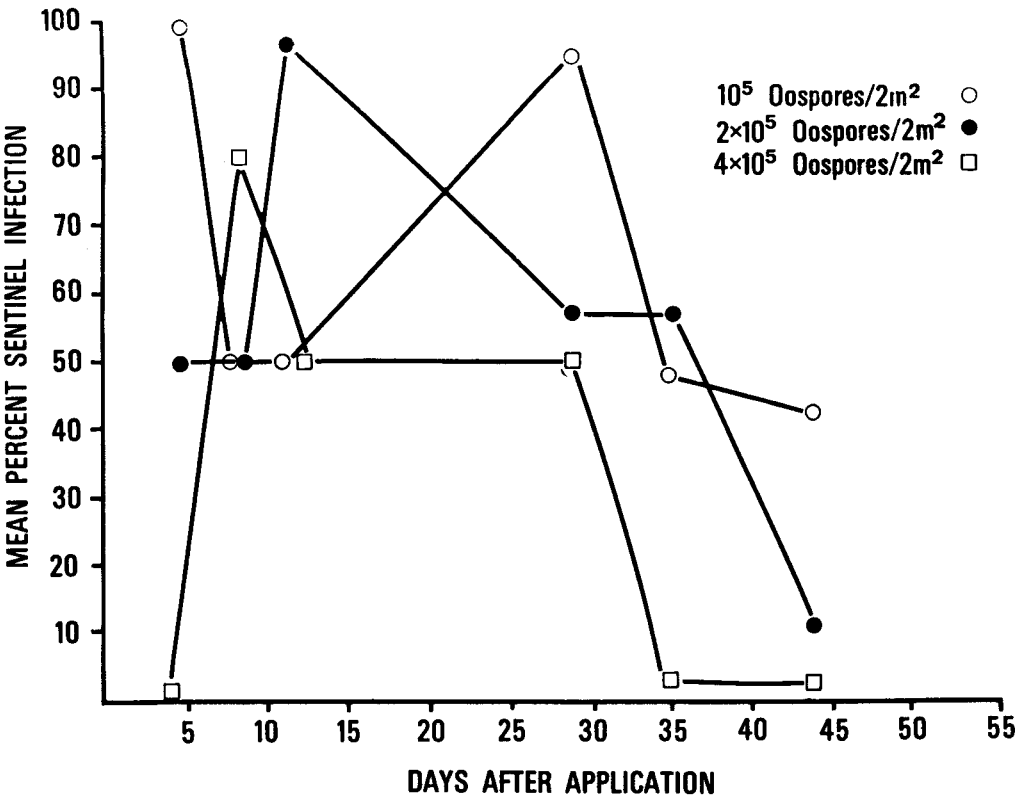

Fig. 3. Infection of *Culex tarsalis* sentinel larvae: Ground applications of oospores. Each point is the percent infection of larvae from 2 sentinel cages.

fermentor-grown cultures of the fungus are ca. 5×10^7 viable oospores/liter (Kerwin et al. 1986). Increased understanding of the physiological and biochemical processes underlying oospore formation, maturation and activation should allow gradual increases in oospore yields of up to 2 orders of magnitude, which is probably necessary for commercially viable production of the fungus.

The major impediment to full exploitation of *L. giganteum* for multi-hectare field control of mosquitoes is the current inability to regulate oospore activation. From observations in the laboratory of different lots of oospores, even after several months of asynchronous germination, less than 20% of the viable spores have germinated. In some larval breeding habitats asynchronous germination over several months is desirable, e.g. when the target species are multivoltine like *Cx. tarsalis* or *An. freeborni* which breed in the rice fields of California's Central Valley. Control of floodwater species such as *Aedes vexans* Meigen or *Psorophora columbiae* (Dyar and Knab) would be minimal using existing oospore preparations. Even in instances where asynchronous germination is a desirable characteristic, higher levels of oospore germination would decrease the amount of sexual stage that would have to be applied to achieve a given level of control. An ideal formulation would have a high initial level of activation to reduce the initial larval population and maximize recycling of the asexual stage. This initial activity would then be followed by gradual and continuous germination of the remaining spores over the rest of the breeding season.

Factors affecting oospore germination among oomycetous fungi are diverse, variable and not well understood (Ribiero 1978, Ruben et al. 1980, Förster et al. 1983). It is likely that all steps of oospore formation including source and maintenance of inoculum, nature of the growth medium, time and method of desiccation and treatment prior to or at the time of activation will affect subsequent spore germination. Major modifications in fermentation media are now being made in response to recent information on in vitro production of oospores by 2 sexually recalcitrant strains of *L. giganteum*, the Louisiana and North Carolina isolates (Kerwin and Washino, unpublished ob-

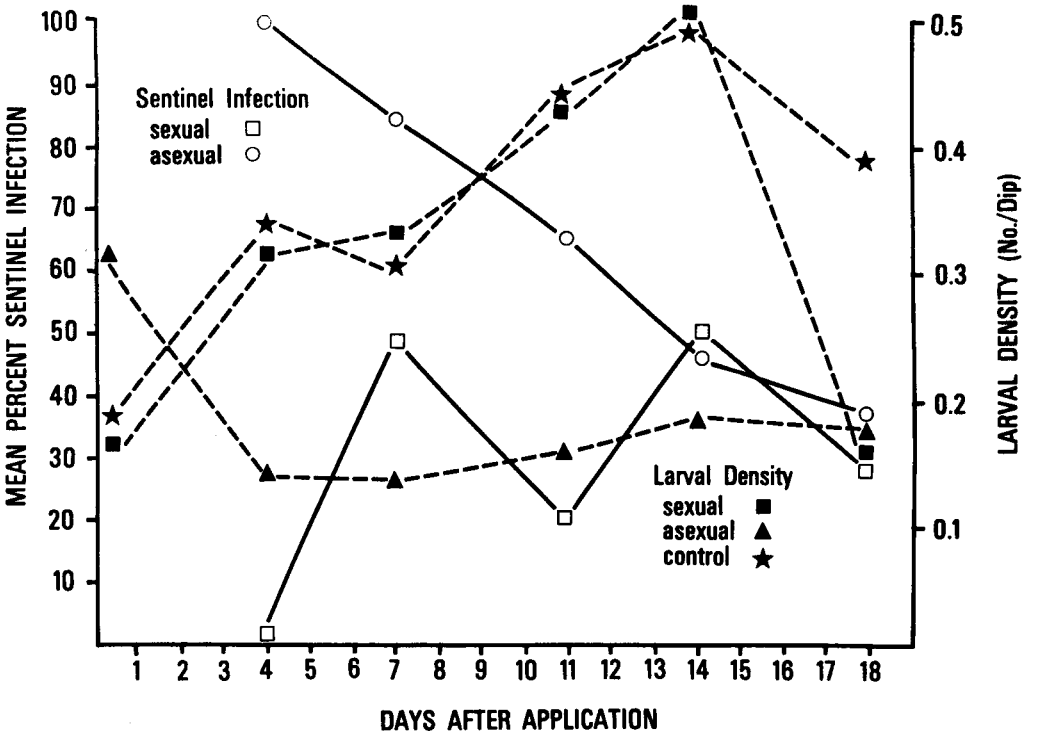

Fig. 4. Infection of *Culex tarsalis* sentinel larvae and density of indigenous larvae (*Culex tarsalis* and *Anopheles freeborni*): Aerial applications of *L. giganteum*. Data referring to percent sentinal infection is based on larvae from 15 sentinel cages. Data referring to larval density is the average of 400 dip samples.

servations). Possibly these growth medium alterations will allow greater control of the germination of the resulting oospores.

These aerial trials were initiated primarily to give an indication of possible problems which might be encountered in the extensive trials planned for 1986. The relatively crude formulation described in this paper is not recommended for general use. With low pressure spray systems becoming routine for the application of *Bacillus thuringiensis* var. *israelensis* (serotype H-14) by a number of mosquito abatement districts, implementation of a comparable scheme for *L. giganteum* would appear to be ideal. This possibility is being actively evaluated in conjunction with local mosquito control agencies.

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