GROUND AND AERIAL APPLICATION OF THE ASEXUAL STAGE OF *LAGENIDIUM GIGANTEUM* FOR CONTROL OF MOSQUITOES ASSOCIATED WITH RICE CULTURE IN THE CENTRAL VALLEY OF CALIFORNIA[1]

JAMES L. KERWIN AND ROBERT K. WASHINO

Department of Entomology, University of California, Davis, CA 95616

ABSTRACT. A series of ground and aerial applications of *Lagenidium giganteum*, a facultative fungal parasite of mosquito larvae, was made in rice fields and associated habitats in the Sacramento Valley, CA. Initial trials using ground applications of the fungus in 400 m^2 plots indicated that asexually competent mycelium from 30 liters of fermentation beer per hectare was sufficient to control *Culex tarsalis* in rice field habitats. Two multi-hectare applications using a Micronair Atomizer® were made at rates of mycelium from either 20 or 30 liters of fermentation beer per hectare. The lower application rate resulted in 40% confirmed infection of *Cx. tarsalis* and *Anopheles freeborni* sentinel larvae, while the higher application rate resulted in greater than 90% initial mortality of sentinel *Cx. tarsalis* and *An. freeborni* and 65% *Aedes melanimon* sentinel mortality. This was accompanied by a 10-fold decrease in indigenous populations of the 2 former species.

INTRODUCTION

The continued development of resistance by mosquitoes to many of the commonly used insecticides (Georghiou and Saito 1983, Brown 1986) requires the introduction of new insecticides, revised control strategies and the use of biological agents. Among the more promising microbial agents for biological control of mosquitoes is the oomycetous fungus *Lagenidium giganteum* (McCray 1985, Lacey and Undeen 1986).

Oospores, the sexual stage of *L. giganteum*, are dormant propagules resistant to desiccation and abrasion, and are stable for at least 7 years (Kerwin et al. 1986). These properties make the sexual stage ideal for large scale commercial production and field application; however, continued problems with spore activation have impeded larger scale field tests (Kerwin and Washino 1986a, Kerwin et al. 1986).

An initial aerial trial using the asexual stage of *L. giganteum* in 1985 (Kerwin and Washino 1986a) documented high sentinel mortality and persistent reduction of the indigenous larval population following application of mycelium from 110 liters of fermentation beer per hectare. This data suggested that previous applications of this stage of the fungus had used excessive amounts of mycelium to achieve desired levels of control (Kerwin et al. 1986). To demonstrate the technical feasibility of using *L. giganteum* for operational field control of larvae, a series of tests culminating in 2 multihectare aerial applications of the asexual stage of the fungus were undertaken in 1986.

MATERIALS AND METHODS

Source, Maintenance and Culture of the Fungus: The California strain of *L. giganteum* (ATCC 52675) was used in these investigations. The isolate was maintained in liquid shake culture by successive transfers of zoospore suspensions in SEX/C medium (g/liter deionized water): 1.5 Ardamine pH autolyzed yeast extract (Desmo Chemical Corporation, Elmsford, NY), 1 glucose, 0.05 cholesterol, 1 hydrolyzed lactalbumin (Sigma), 0.5 dehydrated egg yolk (Sigma), 2 mM calcium, 1 mM magnesium, 1 ml corn oil (Sigma), pH 6.5. Maintenance cultures were grown in 100 ml of medium in cotton-stoppered 500 ml Erlenmeyer flasks at 120 rpm on a rotary shaker for 5–7 days under ambient temperature (23 ± 3°C) and light conditions. Cultures used for inoculation of fermentation flasks were grown in 1 liter of SEX/C medium in 2800 ml Fernbach flasks under these same culture conditions.

For field trials the fungus was grown in 12 liters of media (14 liter tanks) in a New Brunswick Scientific Microferm MF 214 fermentor or in 90 liters of media (130 liter tank) in a NBS Fermacell fermentor, model F-130. The smaller tanks were inoculated with 500 ml of 3 to 7 day old SEX/C cultures and the larger tank with 3 liters. Ground applications and the initial aerial trial used mycelium cultured in 12 liter tanks containing the following medium (g/12 liters distilled water): 15 Ardamine pH, 15 glucose, 5

[1] These trials were performed under restrictions outlined by a special use permit from the Sacramento National Wildlife Refuge Complex. To facilitate use of this fungus for operational mosquito control, detailed mammalian and nonmammalian safety tests performed according to the United States Environmental Protection Agency guidelines have been submitted to the EPA for consideration as a basis for registration of *Lagenidium giganteum* with state and federal agencies.

proflo cottonseed extract (Traders Protein Division, Buckeye Cellulose Corporation, P. O. Box 8307, Memphis, TN), 7 dehydrated egg yolk, 4 $CaCl_2 \cdot 2H_2O$, 2 $MgCl_2 \cdot 6H_2O$, 0.8 cholesterol, 12 ml wheat germ oil (Sigma), pH 7.0. For the second aerial trial the medium in the 12 liter tanks consisted of (g/12 liters distilled water): 20 Ardamine pH, 15 glucose, 10 proflo cottonseed extract, 15 dehydrated egg yolk, calcium and magnesium salts as above, 0.8 cholesterol, 15 ml wheat germ oil, 5 ml proflo oil (Traders Protein Division), pH 7.0. The 130 liter tank was used to produce some of the mycelium used in the second aerial trial and consisted of (g/90 liter industrial water): 200 glucose, 150 Ardamine pH, 60 dehydrated egg yolk, 40 proflo cottonseed extract, 10 cholesterol, 40 $CaCl_2 \cdot 2H_2O$, 10 $MgCl_2 \cdot 6H_2O$, 80 ml wheat germ oil, 40 ml proflo oil, pH 7.0. The smaller tanks were run at 23°C, 150 rpm, and $3.5 - 4.0 \times 10^3$ cc air/min. The larger tank was operated at 28°C, 150 rpm and 2.5 ft^3/min.

When necessary to obtain sufficient material for the aerial trials, mycelium from 5 day old fermentation cultures was filtered onto 100 micron nylon mesh cloth, resuspended in a small volume of distilled water and stored at 15°C until the day of application.

Ground Application of the Asexual Stage: Field trials were carried out at the Colusa National Wildlife Refuge (NWR) in a rice field maintained for migratory birds. Several 400 m^2 plots (20 × 20 m) were staked and a measured amount of the fungus was applied using a hand-pumped backpack sprayer. Other plots were treated using fungus mixed as a slurry with damp corn cobs and disseminated by hand. Initial monitoring with sentinel *Cx. tarsalis* Coquillett mosquito larvae held in plastic sentinel cages with nylon mesh sides and bottom (Case and Washino 1979) confirmed the absence of *L. giganteum* prior to treatment. Each plot was monitored for indigenous larval populations using standard 1 pint (0.47 liter) sampling dippers, 50 dips per plot, on the day of application and 7 days post-application. Further monitoring was not warranted due to the very low indigenous larval population (less than 0.01 larva/dip) and precluded the necessity for control plots. Each 400 m^2 plot was monitored for fungal activity using 5 sentinel cages per plot on day 0 and at various intervals following treatment. Each cage was stocked with twenty-five 1st-2nd instar laboratory-reared *Cx. tarsalis* (Davis strain) and collected 3 days after their introduction into the field. Dead larvae were examined for infection in the laboratory and removed from the collection cups to minimize recycling. Sentinel larvae were subsequently examined for 2–3 days to assess latent infection by *L. giganteum.*

Maximum/minimum thermometers were placed in some plots to record the temperature range during the trials. Water samples were taken and chemical characteristics analyzed by the University of California, Davis, Cooperative Extension Water Analysis Laboratory.

Aerial Application of the Asexual Stage: The initial aerial trial was undertaken in the same field as the ground treatments. A 2 hectare plot, ca. 60 × 330 m, was staked and absence of the fungus confirmed using sentinel cages. Indigenous larval populations were sampled on days 0 and 7 of the trial, with 3 operators each taking 200 dips parallel to each other along 200 m of the long axis of the plot. Two separate transects were dipped by each operator, each 100 m long beginning at either end of the treated plot. Adjacent transects were separated by 15 m. The very low native larval mosquito population again precluded the need to monitor a control plot.

Fifty sentinel cages, each containing fifteen 1st-2nd instar *An. freeborni* and fifteen 2nd-3rd instar *Cx. tarsalis*, were placed in 5 rows of 10 cages each perpendicular to the long axis of the plot. The rows were spaced at either end of the plot, 75 m from the east end of the plot and 75 m and 150 m from the west end. Eight of the cages in each row were spaced evenly within the boundaries of the plot and the other 2 were placed 10 m outside each boundary. Sentinels were placed, collected and monitored as described for the ground applications.

Mycelium was applied using a Micronair Atomizer® calibrated to give a 30 m swath width. It was mounted on a Paunee 235 aircraft. The mycelium was suspended in distilled water and applied at 32 oz per acre (0.94 liter per hectare). Two passes were made over each of 2 transects to deliver a total of 64 oz of mycelial suspension per acre. Mycelium from 20 liters of fermentation beer was applied per hectare, equivalent to ca. 5×10^{10} cells per hectare. Over 95% of the cells released zoospores from the material used in this test when monitored under laboratory conditions. Wind was 4–5 knots from the SW at the time of application.

A second aerial trial in the Colusa NWR was set up in a several hundred acre pond containing primarily alkali bulrush, *Scirpus* sp. which supported a relatively high indigenous larval population of *Cx. tarsalis* and *Anopheles freeborni* Aitken. A 4 hectare plot, 90 × 660 m, was staked and the absence of fungus confirmed using sentinel mosquitoes. Two 300 m transects, 30 m and 60 m from the west edge of the plot, were dipped to monitor the native larval population. A total of 600 dips were made on days 0, 3 and 10 of the trial and compared to the indigenous population in a control plot in the same field 50 m from one edge of the treated site. Larvae

dipped from the treated field were returned to the laboratory and held for 3 days to assess percent infection. Indigenous larval density reported from the treated field was generated by subtracting the number of infected larvae as monitored in the laboratory from the total number of larvae dipped from the treated field.

Fifty sentinel cages stocked as described for the initial aerial trial were placed in the field ca. 15 m from the west edge along the entire 660 m length of the plot. In the initial trial sentinel cages were stocked and the tops placed on before application of the fungus. For the second aerial evaluation the tops were left off until after the mycelium had been flown on to allow the fungus to be dropped directly into the cages. Sentinel placement, collection and monitoring protocols were as described above. One day after the field had been treated a breeding site containing a high density of *Aedes melanimon* Dyar was found at Gray Lodge State Wildlife Area. Larvae were collected from this site, primarily early second instar, and 50–75 larvae placed in each of 50 sentinel cages arranged parallel to the first set.

Using different growth media and a larger fermentor as detailed previously, mycelium from 30 liters of fermentation beer was applied per hectare, ca. 5.5×10^{10} cells per hectare. Wind was variable, 2–12 knots from the SE, at the time of application. Water temperature and chemical characteristics were documented as described for previous trials.

Anopheles freeborni and *Cx. tarsalis* sentinels were removed 48 hours after the second aerial application of the fungus due to very high initial mortality. *Aedes melanimon* sentinel larvae were also removed after 48 hours exposure in the treated field, i.e., 3 days after the aerial application. Subsequent sentinel exposure and monitoring was performed over 3 day periods as described previously.

Data are reported as means or means ± 1 standard deviation as appropriate. The number of samples n refers to the number of sentinel cages used to generate a single data point, with each sentinel cage stocked with larvae as described previously.

RESULTS

Ground Application of the Asexual Stage: To establish lower limits of application, a series of 400 m^2 plots were treated over a 45 day period and monitored using sentinel larvae. Due to continued restrictions on the application of *L. giganteum*, these tests were restricted to rice fields maintained for migratory birds at the Colusa NWR, which unfortunately supported a very low density of mosquito larvae. Density varied from 0 to 0.01 larva/dip, which is too low to support appreciable recycling of the fungus.

The water temperature range during the course of these trials was 19–35°C. Pertinent water characteristics were well within the range of conditions tolerated by the fungus and suitable for the induction of asexual zoosporogenesis (Table 1).

Even at the lowest rate used for these initial trials, high levels of sentinel *Cx. tarsalis* mortality was documented (Table 2). Sentinel infection dropped appreciably 10 days postapplication at all rates tested except at the 30 liter per hectare plot, which exhibited 40–50% infection 14 and 17 days after application. The formulation applied in these plots contained an oospore density equivalent to ca. 1.6×10^6 oospores per hectare and, based on laboratory observations, probably did not germinate during the period that these plots were monitored.

Aerial Trials: The initial aerial trial applied mycelium from 20 liters of fermentation beer per hectare, equivalent to 5.0×10^{10} asexually competent cells per hectare. This resulted in 30–40% initial infection of *An. freeborni* and *Cx. tarsalis* sentinels (Table 3), with appreciable reduction in fungal activity after day 10. The estimate of effectiveness of this application was based solely on sentinel data and may have under-estimated the activity of the fungus as discussed below.

Indigenous larval density varied from 0 to 0.01 larva/dip. The temperature range during the monitoring period was 19–32°C and water quality was within the range tolerated by the fungus (Table 1). Asexual sporulation in water from the

Table 1. Water characteristics of sites treated with the asexual stage of *Lagenidium giganteum.*

Characteristic[1]	Site description		
	Ground applications	Aerial #1	Aerial #2
Ca (me/liter)	1.2	1.2	1.4
Mg (me/liter)	1.7	1.5	1.6
Na (me/liter)	2.17	2.17	3.04
Cl (me/liter)	0.21	0.17	0.82
B (ppm)	0.51	0.52	0.34
$CO_3 + HCO_3$ (me/liter)	4.4	4.8	4.9
SO_4-S (me/liter)	0.01	0.01	0.18
NO_3-N (ppm)	0.80	0.80	0.41
NH_4-N (ppm)	0.10	0.10	0.10
pH	7.0	6.9	7.6
EC (millimicroohms/cm)	0.46	0.46	0.59
PO_4-P (ppm)	0.28	0.23	0.01

[1] EC - electrical conductivity; me - milliequivalents. All water tests were performed by the University of California, Davis, Cooperative Extension Water Analysis Laboratory. Units reported are those routinely used by the laboratory to summarize their data.

Table 2. Percent infection of sentinel *Culex tarsalis* larvae following ground application of the asexual stage of *Lagenidium giganteum* in 400 m^2 rice field plots. Each data point is based on 3 plots, each containing 5 sentinel cages, 25 larvae per cage.

	% sentinel infection[b] (mean ± 1 SD)					
	Days after application					
Formulation/rate[a]	0	3	7	10	14	17
liquid-30 liters/hectare	0	100	78 ± 12	18 ± 26	48 ± 16	40 ± 27
liquid-50 liters/hectare	0	100	74 ± 7	21 ± 18	9 ± 11	9 ± 17
liquid-100 liters/hectare	0	100	69 ± 14	26 ± 32	4 ± 11	0
granular-100 liters/hectare	0	69 ± 14	82 ± 21	60 ± 37	0	0
liquid-500 liters/hectare	0	100	78 ± 18	50 ± 23	0	0

[a] Liquid: aqueous suspension of mycelium in rice field water applied using a backpack sprayer. Granular: corn cob slurry applied by hand. Rates are presented as the amount of fermentation beer used to produce sufficient mycelium for the various applications.
[b] Day 0: n = 5. All other days: n = 15.

Table 3. Percent infection of sentinel and indigenous *Culex tarsalis* and *Anopheles freeborni* larvae following aerial application of the asexual stage of *Lagenidium giganteum*. Each data point for sentinel infection is based on 50 sentinel cages, each containing 15 larvae of each species. Indigenous larval infection is based on percent infection as monitored in the laboratory of larvae collected from 600 dip samples.

	% sentinel infection[a] (mean ± 1 SD)					
	Anopheles freeborni			*Culex tarsalis*		
Day after application	Aerial #1[b]	Aerial #2[b]		Aerial #1[b]	Aerial #2[b]	
0	0	0		0	0	
2	—	95 ± 6		—	98 ± 3	
3	32 ± 23	—	(58)	46 ± 33	—	(84)
6	25 ± 23	—		44 ± 29	—	
7	—	28 ± 31		—	35 ± 33	
9	30 ± 19	—		25 ± 29	—	
10	—	15 ± 21	(44)	—	34 ± 37	(78)
12	25 ± 22	—		14 ± 26	—	
15	10 ± 17	—		12 ± 24	—	

[a] Day 0: n = 5. All other days: n = 50. For day 2 of the second aerial trial the data refer to percent mortality. All other data points are percent infection. Figures in parentheses are infection rates in indigenous larvae.
[b] Aerial #1: First aerial trial. Aerial #2: Second aerial trial. Experimental protocols are detailed in the text.

treated site was induced in 95% of the cells from a subsample of the applied material as monitored in the laboratory.

A second aerial trial was carried out in mid-September when a field on the Colusa NWR supporting a significant mosquito population was finally discovered. Application of 5.5×10^{10} asexually competent cells per hectare (mycelium from 30 liters of fermentation beer) resulted in a 7-fold reduction in the native population of *An. freeborni* and a 10-fold reduction in indigenous *Cx. tarsalis* (Table 4). A 130 liter fermentor containing 90 liters of growth medium was used to grow most of the mycelium for this trial. The cell density produced in this tank was less than that from the smaller tanks, which accounts for the relatively small increase in the number of cells applied per hectare despite increasing the equivalent amount of fermentation beer applied from 20 to 30 liters per hectare for the second aerial trial.

An unexpected rapid death of sentinel larvae in the treated plot following 48 hours exposure in the field occurred during the initial monitoring period (Table 3). *Aedes melanimon* sentinel mortality after 48 hours exposure in the field was 65 ± 28% (n = 50). For the first monitoring period only, the points correspond to sentinel mortality rather than confirmed infection. Infection could not be confirmed in the majority of dead sentinel larvae (21% confirmed infection for *Cx. tarsalis*, 3% for *An. freeborni* and 4% for

Table 4. Indigenous larval density monitored during the second aerial application of the asexual stage of *Lagenidium giganteum*.

	Number of larvae/dip[a]			
	Control		Treated	
Days after application	*Anopheles freeborni*	*Culex tarsalis*	*Anopheles freeborni*	*Culex tarsalis*
0	0.40	2.1	0.36	0.25
3	1.3	1.8	0.05	0.02
10	0.82	3.4	0.08	0.06

[a] Larval density is based on 100 dip samples for the control plot and 600 dip samples for the treated plot.

Ae. melanimon) due to rapid decay of the dead larvae and the presence of a very high density of saprophytic fungi, algae and protists. This phenomenon has frequently been noted in the laboratory in rice water samples following massive zoospore encystment on susceptible mosquito larvae, which are apparently killed by systemic shock.

In contrast to the high initial mortality in the treated plot, mortality in 3 sentinel cages in the control plot was 3% for *Cx. tarsalis*, 7% for *An. freeborni* and less than 1% for *Ae. melanimon* (one sentinel cage with more than 125 larvae).

Except for the initial monitoring period, infection of indigenous larvae exceeded that of the corresponding sentinel larvae. This suggests that sentinel infection was perhaps an underestimation of the effectiveness of the first aerial application (Table 3). Independent monitoring of the first aerial trial by local mosquito abatement personnel using 1-pint solid plastic sentinel cages without tops which were removed from the rice field immediately after aerial application of the fungus, documented greater than 70% infection of sentinel *Culex pipiens* Linn., which is very similar to *Cx. tarsalis* in its susceptibility to infection by *L. giganteum*, within 3 days after returning the cups to the laboratory.

Temperatures ranged from 18 to 24°C during monitoring of this field. Water characteristics of the treated field, listed in Table 1, were conducive to asexual zoosporogenesis. Greater than 98% of the cells were asexually competent as monitored in the laboratory.

DISCUSSION

Field trials described in this report document the feasibility of using fermentor-grown cultures of the asexual stage of *L. giganteum* for multihectare control of mosquito larvae. Previously described methods for producing the asexual stage of the fungus in solid and liquid media (Domnas et al. 1982, Jaronski et al. 1983, Kerwin and Washino 1983, Jaronski and Axtell 1984) are labor-intensive, use relatively expensive culture techniques and are amenable to small scale field application. Fermentation tanks can vary in size up to tens of thousands of liters and variations of the media described in this study can be scaled-up to accommodate larger scale production.

The media used in these studies were not systematically developed to optimize asexual zoosporogenesis but were the result of efforts to increase yields of the sexual (oospore) stage which yielded few oospores but great numbers of asexually competent cells. Substitution of less expensive components for nutrients such as dehydrated egg yolk in the media, e.g., peptones available in bulk quantities from a variety of sources, can readily be made with increased yields of asexually competent cells in many cases. Requirements for production of the asexual stage are much less demanding than those for oospore production (Kerwin and Washino 1986b, Kerwin et al. 1986) and high yields can be obtained using a variety of fermentation media.

Application rates of mycelium from 20 to 30 liters of fermentation beer per hectare approach the production requirements for field control of mosquito larvae using the microbial insecticide *Bacillus thuringiensis* var. *israelensis* (H-14) which has been the subject of intensive investigation by both industrial and academic laboratories. The range of volume requirements to produce sufficient quantities of this bacterium to treat 1 hectare of mosquito breeding habitat averages from 5 to 10 liters using current technology (Terry Couch, personal communication). The ability to demonstrate field control of mosquito larvae by *L. giganteum* using application rates comparable to this commercially available product which has undergone years of extensive development indicates its potential as a commercially viable alternative to chemical control. The ability of the fungus to recycle and persist in mosquito habitats for months or even years (Jaronski and Axtell 1983, Fetter-Lasko and Washino 1983, Kerwin and Washino 1986a) further enhances the cost-effective implementation of *L. giganteum* in integrated control strategies.

Aerial application of this relatively fragile stage of the fungus using low pressure spray systems appears to be an effective method of dissemination, requiring minimal formulation or special handling. It proved to be preferable to the corn cob formulation used for the initial aerial trials in 1985 (Kerwin and Washino 1986a).

The disadvantages of using this stage in the life cycle of *L. giganteum* are its inability to withstand desiccation or abrasion and its relatively short stability in storage, ca. 2 to 8 weeks depending on production and storage protocols. Research emphasis continues to be on factors regulating production and activation of the dormant oospore stage of the fungus (Kerwin and Washino 1986b, Kerwin et al. 1986) which has the requisite stability for large scale commercial production.

Use of *L. giganteum* for field control of mosquitoes, especially during initial evaluations in larval breeding habitats not previously treated with the fungus or against species of mosquitoes or even different populations of mosquitoes not previously exposed to the fungus, requires attention to a number of environmental and host-mediated factors which can affect larval infec-

tion. Besides the documented limitations of water temperature, salinity, organic content and related factors (Jaronski and Axtell 1982, Merriam and Axtell 1982, Lord and Roberts 1985), a direct test of zoospore induction should be made using the exact formulation of *L. giganteum* which will be applied in the field using water from the site to be treated. Mosquito larvae collected from sites to be treated should be exposed to *L. giganteum* in the laboratory prior to field application to verify susceptibility to parasitism at the approximate rates at which the fungus is to be applied. Although rarely documented, later instars of susceptible populations of some mosquito species are not recognized as suitable hosts by *L. giganteum* zoospores (Kerwin et al., unpublished data).

The strain, source, maintenance, culture and storage of *L. giganteum* all affect its sporulation capacity and pathogenicity to mosquito larvae. Careful documentation and control of the cited factors will optimize routine use of this fungus for mosquito control.

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