ENCAPSULATION OF THE MOSQUITO FUNGAL PATHOGEN *LAGENIDIUM GIGANTEUM* (OOMYCETES:LAGENIDIALES) IN CALCIUM ALGINATE[1]

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ABSTRACT. The asexual stages (presporangial mycelia) of *Lagenidium giganteum* cultured on sunflower seed extract were encapsulated in calcium alginate and remained infective to mosquito larvae, *Culex quinquefasciatus*, after storage at 15°C for up to 75 days. Survival and zoospore release from the encapsulated mycelia persisted for up to 24 days after immersion in water. Encapsulated sexual stages (oospores), held at 23-26°C for up to 35 days or frozen for 8-10 days, were infective to mosquito larvae for up to 48 days after immersion in water and during that time over 50% of the oospores germinated. In outdoor pools, encapsulated mycelia gave 100% control of *Cx. quinquefasciatus* at 6-7 days after treatment while encapsulated oospores gave 100% control at 11 days posttreatment.

INTRODUCTION

The fungus *Lagenidium giganteum* Couch is one of the most promising biological control agents against mosquito larvae (Lacey and Undeen 1986). Although significant improvements have been made in culturing and mass production of *L. giganteum* in the laboratory (Domnas et al. 1982, Jaronski et al. 1983, Jaronski and Axtell 1984, Guzman and Axtell 1986, Kerwin et al. 1986), little emphasis has been placed on methods for formulating the fungal inoculum for application into mosquito-breeding habitats. *Lagenidium giganteum* has been applied using liquid or solid cultures of mycelia (Jaronski and Axtell 1983, Jaronski and Axtell 1984, Guzman and Axtell 1986), or infected mosquito cadavers (McCray et al. 1973). More recently Kerwin and Washino (1986) evaluated fermentor-grown *L. giganteum* mycelia and oospores formulated on corn cob pellets. They reported up to 40% mortality of the asexual stage (mycelia) in the corn cob formulation due to desiccation, mechanical abrasion, and storage for one week prior to application.

Alginate encapsulation has been successful with certain chemicals, plant pathogenic bacteria and fungi (Bashan 1986, Connick 1982, Fravel et al. 1985, Walker and Connick 1983); however, the only entomopathogens that have been encapsulated in calcium alginate are steinernematid and heterorhabditid nematodes (Kaya and Nelsen 1985). If encapsulated *L. giganteum* retained its infectivity, the formulation would be more convenient to store, handle and apply than are the presently used preparations from agar or liquid cultures. Therefore, experiments were conducted to develop a procedure for encapsulation of the asexual (presporangial mycelia) and sexual (oospores) stages of *L. giganteum* in calcium alginate beads and to evaluate the effect of encapsulation on fungal infectivity in laboratory bioassays and preliminary field trials.

MATERIALS AND METHODS

Fungal isolate. Lagenidium giganteum (California strain ATCC Accession No. 52675) was obtained in June 1984 from J. Lord, Boyce Thompson Institute, Ithaca, NY, who obtained the original culture from J. Kerwin, Univ. of California, Davis, CA. The fungus was subcultured weekly on SFE (sunflower seed extract, 2-4 mg/ml soluble protein concentration) for about 18 months prior to culturing in larvae of *Culex quinquefasciatus* Say through ca. 70 serial passages. The fungus was reisolated from infected cadavers onto SFE agar plates containing antibiotics and then subcultured into SFE (6.0 mg/ml soluble protein) liquid. The SFE liquid culture was placed on a shaker at 100 rpm and room temperature (23-26°C), and thereafter subcultured at weekly intervals by transferring zoospores to fresh media. Further explanations of these SFE culturing methods are given by Guzman and Axtell (1986) and Jaronski and Axtell (1983).

Encapsulation of mycelia. After 10 days growth in SFE liquid, the mycelia developed to the presporangial phase (Fig. 1) in which nearly all cells had an oval appearance and were observed to be capable of completing development to sporangia which produced zoospores, the stage infective to mosquito larvae. No sexual stages (oospores) were produced and only trace amounts of undifferentiated mycelia were present. At this stage the mycelia in 100 ml of SFE liquid culture were washed three times with sterile deionized water, resuspended in 100 ml sterile deionized water and homogenized for 10

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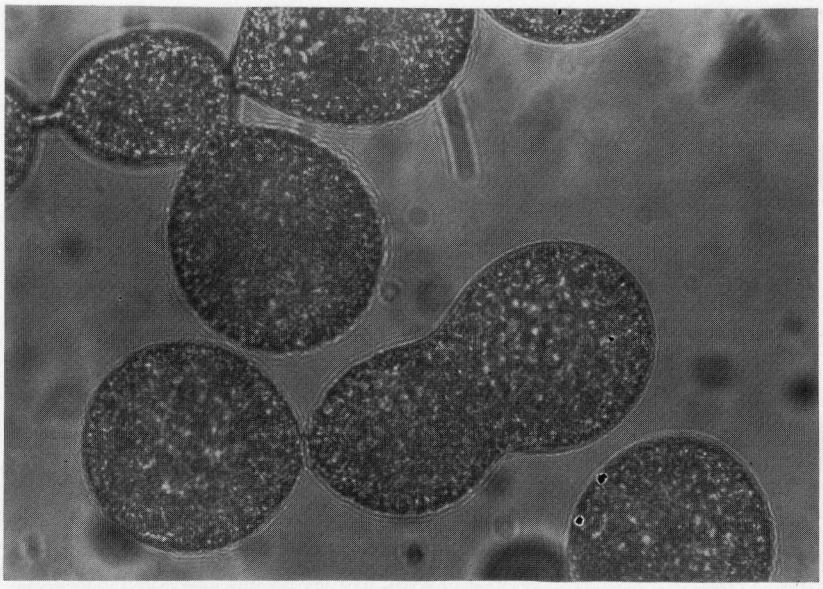

Fig. 1. Presporangia of *Lagenidium giganteum.*

seconds at high speed in a commercial blender. Four hundred ml of sodium alginate solution (6 gm/liter sodium alginate granules, Fisher Scientific Co., Springfield, NJ 07031, cat. no. S-211) were added to the 100 ml of fungal liquid culture resulting in a mixture with a sodium alginate concentration of 4.8 gm/liter. The mixture of suspended mycelia and sodium alginate was agitated continuously with a magnetic stirrer in a reservoir and, by means of tubing and pipettes attached to an opening at the bottom edge of the reservoir, was dispensed drop-wise into 500 ml of a 0.25 M $CaCl_2$ gellant solution (36.8 g/liter $CaCl_2 \cdot 2H_2O$). The drops reacted with the gellant solution to form calcium alginate and rapidly solidify. After 3-10 minutes the capsules were removed and washed in deionized water. The capsules (Fig. 2) were placed in sealed plastic containers and stored at 15°C, unless otherwise stated, until used.

Encapsulation of oospores. Oospores (Fig. 3) were produced in liquid culture with the following composition, which was based on the various media described by Kerwin and Washino (1983, 1986) and Kerwin et al. (1986): 1.2 g autolysed yeast extract (Desmo Chemical Corp., Elmsford, NY), 0.8 g glucose, 0.02 g cholesterol, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.2 g $MgCl \cdot 6H_2O$, 0.25 ml cod liver oil, and 0.6 ml wheat germ oil per 1000 ml deionized water with the pH adjusted to 6.6 with 1N NaOH. Eight 2-liter flasks, each containing 1200 ml of media, were autoclaved, then inoculated with 1-2 ml of zoospore suspension ($1–2 \times 10^4$ zoospores/ml) and placed on a shaker at 100 rpm for 10-15 days. Using this procedure, yields of ca. $2–4 \times 10^4$ viable oospores per ml medium were obtained. Viability was assessed based on the morphological characters described by Kerwin and Washino (1983).

The fungal material from each flask was transferred to a 2-liter graduated cylinder, allowed to settle, and washed 3–5 times at 12-hour-intervals with ca. 1600 ml deionized water. The fungal material was then blended at high speed for 1 minute in a commercial blender and encapsulated as described previously for the mycelia. The resulting capsules (Fig. 2) contained a mixture of undifferentiated mycelia and oospores dispersed throughout the gel matrix. Two batches of capsules were frozen at −15 to −20°C for 8 and 10 days, respectively, in order to kill the mycelia prior to the bioassays for survival of the encapsulated oospores. Preliminary tests demonstrated that frozen capsules containing only mycelia did not cause infection in mosquito larvae. Another batch of capsules was held immersed in deionized water for 5 days prior to the bioassays, and 2 other batches were held at room temperature (23-26°C) in closed containers, for 15 or 35 days prior to bioassay.

Bioassay of encapsulated mycelia. Bioassays were conducted in the laboratory to evaluate the infectivity of *L. giganteum* mycelia (mostly presporangia) after encapsulation in calcium alginate and storage at 15°C for up to 75 days. The first bioassay was designed to test the short term effect of encapsulated as compared to non-encapsulated fungus. There were 4 types of treatment: 1) encapsulated mycelia stored at 15°C for 1 day, 2) free mycelia in deionized water

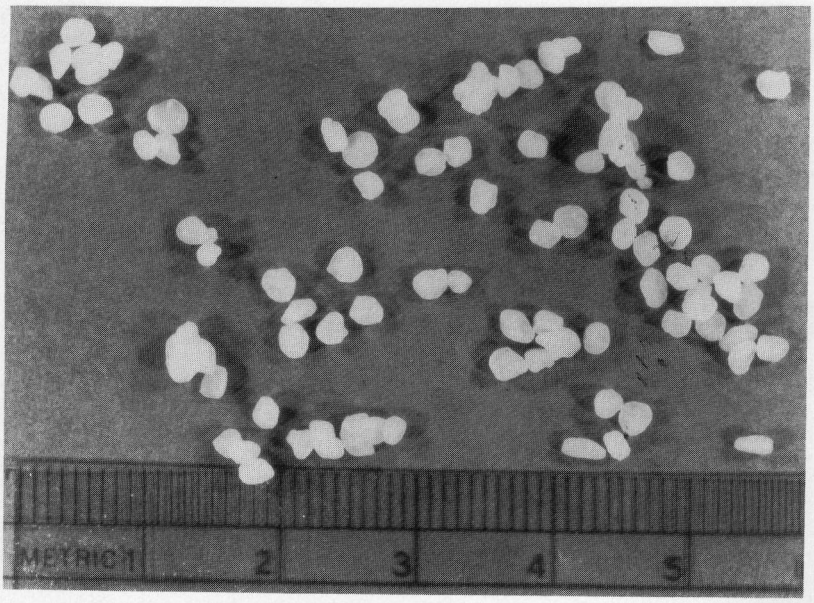

Fig. 2. Calcium alginate capsules containing *Lagenidium giganteum.*

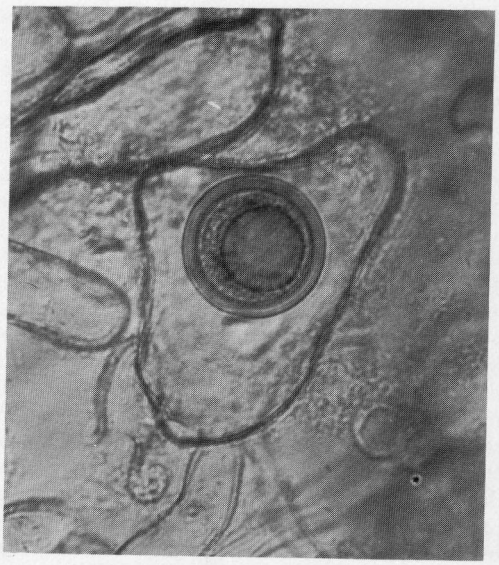

Fig. 3. Oospore of *Lagenidium giganteum*, with well-developed wall and concentric lipid globule (ooplast).

(from the same batch used for encapsulation), 3) SFE agar culture disks (200 mm^2) of mycelia, and 4) motile zoospores produced from SFE agar disks of mycelia immersed in deionized water. The bioassays were conducted using plastic cups (9 cm diam × 6 cm height) containing 100 ml deionized water, 0.25 ml of a liver powder slurry (35 mg/ml). Twenty-five second- or third-instar *Cx. quinquefasciatus* larvae (3-day-old) were added to each cup (3 cups per treatment) at 1, 3, 6, and 12 days after treatment. To eliminate recycling of the fungus in the host larvae, dead individuals were removed twice a day and examined microscopically for evidence of fungal infection. The cups were held at 25°C during the 3-day bioassay period and liver powder slurry (0.25 ml) was added daily to those cups which contained mosquito larvae.

Other bioassays were performed with batch #1 of capsules stored for 19, 40, 53 and 75 days; batch #2 of capsules stored for 8, 22, 42 and 64 days and batch #3 of capsules stored for 8 days. The alginate treatment was 20 capsules per cup in all except the last bioassay, in which dosages of 10, 5 and 1 capsules (8-days-old) per cup were also used.

Bioassay of encapsulated oospores. The bioassay consisted of 6 treatments: 1) Disks (20 mm^2) cut from SFE agar culture plates containing mycelia of *L. giganteum* as a reference to compare encapsulated material with a standard mycelial formulation (Jaronski and Axtell 1984, Guzman and Axtell 1986); 2) encapsulated oospores and mycelia held in deionized water for 5 days at room temperature (23-26°C); 3) and 4) capsules which were held in a closed container for 7 days at room temperature and then frozen at −15 to −20°C for 8 to 10 days, respectively, before thawing for the bioassays; and 5) and 6), capsules held in a closed container at room temperature for 15 and 35 days, respectively, under relatively dry conditions. Tissue culture plates (Costar, Cambridge, MA) were used for these assays, with one capsule of a treatment (or one 20 mm^2 agar disk) placed in 2.5 ml

deionized water into each of the 24 wells (16 mm diam.) of a plate. The covered plates were held in an incubator at 25 ± 1°C for 1, 3, 6, 12, 24 or 48 days and then one second-instar larva of *Cx. quinquefasciatus* and one drop of a dilute suspension of liver powder were added to each well. The plates were then held at 25°C for 2 days after which the number of infected larvae in each plate was determined by microscopic examination. There were 3 plates (72 larvae) for each treatment-period combination. Water levels in individual wells were corrected for evaporation when necessary by adding deionized water at weekly intervals.

Quantification of treatments. The average number of *L. giganteum* presporangia per capsule was determined by sampling 5 capsules from each batch. Each bead was gently flattened onto a glass plate counting chamber (13 mm diam × 0.3 mm deep, ceramic ring slide, Fisher Scientific Co., cat. no. 12-568-30) using a microscope slide coverslip. The diameter of the flattened alginate disk was recorded, and the number of cells per 3.14 mm^2 microscopic field was counted on 5 sections of the disk at 100× magnification. The maximum potential number of zoospores that could be produced from each alginate capsule was estimated by multiplying the number of cells per capsule by 15, the average number of zoospores per sporangium.

The number of zoospores in suspensions used in the bioassays was determined by a method similar to one described by Lord and Roberts (1985). Two ml of zoospore suspension were placed in 60 mm diam. plastic petri dishes, and then 3 ml deionized water were added to cover the bottom surface of the dish. Zoospores encysted and germinated on the bottom of the dish within 48 hours. The number of germinated zoospores was counted in five 3.14 mm^2 microscopic fields and the number per ml calculated. For the treatment using free mycelia, the cells were counted in a chamber of a ceramic ring slide described previously. The chamber (0.045 ml) was filled with a sample of well-mixed mycelia and the presporangia counted in 5 microscopic fields. Determination of the number of zoospores produced from the agar disk treatments was by the procedures described by Guzman and Axtell (1986).

Field trials. Two field trials using encapsulated *L. giganteum* were conducted in the vicinity of Raleigh, NC, in June and August 1986. Nine plastic wading pools (1.2 m diam. × 0.2 m deep) were used to simulate stagnant water mosquito breeding habitats. The bottom of each pool was covered with 2–4 cm of sand and a sparse layer of forest litter and filled to a depth of 10–15 cm with water from an adjacent pond. Each pool was enclosed in a 1.8 m square × 1.8 m high cage (20-mesh screen) to prevent natural oviposition. Egg rafts of *Cx. quinquefasciatus* (6 per pool) were added daily during the trials. To assure adequate nutrients for the mosquito larvae, 60 ml of a chicken feed slurry (170 g laying mash/1 liter water) were added to each pool twice a week.

Larval and pupal abundance were determined by standard dipping (350 ml dipper) with 3 dips taken per pool before treatment and at 1–3 day intervals after treatment with alginate capsules of *L. giganteum*. Water quality was determined based on measurements of conductivity, dissolved oxygen, temperature and pH performed at the site on the day of fungal application. In the first trial, encapsulated mycelia was applied to the pools on June 30, 1986, in 4 dosages (220, 440, 890 and 1,780 capsules per pool; 2 pools per dose) and one pool was left untreated. The capsules were prepared 32 days prior to use and stored at 15°C, as described previously but using a sodium alginate concentration of 4 g/liter. The estimated number of presporangia per capsule was 1,800. Prior to the second trial the pools were emptied, cleaned and dried in the sun for several days before stocking with sand, pond water and egg rafts. In the second trial the fungus was applied on August 21, 1986, at one dose (200 capsules per pool) using either mycelia encapsulated using 8, 16 and 20 g/liter sodium alginate or oospores encapsulated using 8 g/liter sodium alginate. The estimated no. of presporangia or oospores per capsule were 921 and 3,000, respectively. The capsules containing presporangia were stored for 16 days at 15°C prior to use and the capsules containing oospores were stored frozen (−15°C) for 14 days prior to use.

RESULTS

Production of alginate-encapsulated *L. giganteum* mycelia was a relatively simple procedure but the size of the capsules and number of presporangia per capsule varied considerably among the batches (Table 1). There was no noticeable fungal development within the capsules during storage at 15°C or in capsules held at room temperature (ca. 25°C). After immersion of the capsules in deionized water for 8–24 hours at room temperature zoospore production from the encapsulated presporangia was observed. Discharge tubes grew from the sporangia towards the surface of the capsules; vesicles were formed outside the capsule, and zoospores were discharged within 15–30 minutes of vesicle formation.

Bioassays of encapsulated mycelia. The bioassay data (Table 2) indicated that *L. giganteum* presporangia remained viable in calcium algi-

Table 1. Mean relative size of calcium alginate capsules and mean number of presporangia of *Lagenidium giganteum* per capsule[1] for 3 batches of capsules used in larval bioassays.

Batch no.	Diameter (mm) of flattened capsule mean ± SD	Sporangia per capsule mean ± SD	Estimated no. of zoospores/ml released[2]
1	4.1 ± 0.5	325 ± 15	975
2	5.9 ± 0.5	929 ± 95	2787
3	3.6 ± 0.4	428 ± 28	1284

[1] Each value is mean of 5 capsules from each batch.
[2] With 20 capsules/100 ml H_2O, assuming 15 zoospores per vesicle.

Table 2. Mean percent infection of 2–3 day-old *Culex quinquefasciatus* larvae in bioassays of *Lagenidium giganteum* mycelia (presporangia) encapsulated in calcium alginate.

Treatment (no. of capsules per cup)	Storage time (days) of capsules at 15°C	Percent larval infection at days posttreatment[1]				
		1	3	6	12	24
		Assay no. 1				
Capsules—Batch 1 (20)	1	100	100	100	53.3	—
Agar culture disk	4	100	100	40.0	17.3	—
Motile zoospores	—	77.3	100	2.7	1.3	—
Free presporangia	1	100	100	40.0	16.0	—
		Assay no. 2				
Capsules—Batch 1 (20)	19	100	78.7	37.3	6.7	—
Capsules—Batch 2 (20)	8	100	100	100	84.0	—
Motile zoospores	—	36.0	12.0	0	0	—
		Assay no. 3				
Capsules—Batch 1 (20)	40	33.0	35.0	1.0	2.0	3.0
Capsules—Batch 2 (20)	29	100	100	100	100	61.3
		Assay no. 4				
Capsules—Batch 1 (20)	53	100	99.0	72.0	80.0	89.7
Capsules—Batch 2 (20)	42	100	100	100	99.0	77.0
		Assay no. 5				
Capsules—Batch 1 (20)	75	100	94.7	29.3	84.0	58.7
Capsules—Batch 2 (20)	64	96.0	100.0	98.7	98.7	62.7
Capsules—Batch 3 (20)	8	100	97.3	81.3	76.0	52.0
Capsules—Batch 3 (10)	8	100	81.3	60.0	76.0	16.0
Capsules—Batch 3 (5)	8	97.3	42.7	28.0	37.3	2.7
Capsules—Batch 3 (1)	8	58.7	4.0	5.3	24.0	2.7

[1] Each value is mean of 3–4 replicate cups of 25 larvae each.

nate capsules and the residual activity (i.e., ability to infect mosquito larvae) was greater than when free (not encapsulated) presporangia, zoospores or agar disks of mycelia were used. In the first bioassay there was 100% infection of 3-day-old larvae at 1, 3 and 6 days treatment with the encapsulated fungus. After 12 days, there was 53% infection. Infection rates in cups treated with capsules were initially (1 and 3 days posttreatment) similar to those treated with agar culture disks, free presporangia or motile zoospores but were higher at 6 and 12 days posttreatment. The overall number of zoospores produced in these treatments was estimated at 975, 891, 225 and 3,905 zoospores/ml for capsules, agar disks, motile zoospores and free presporangia, respectively.

In the second bioassay using two different batches of calcium alginate capsules, there were relatively high infection rates at 6 days posttreatment. The capsules had been stored at 15°C for 8 and 19 days and yielded ca. 2,787 and 975 zoospores/ml, respectively. The 8-day-old capsules produced 84% mortality at 12 days posttreatment. The treatment with motile zoospores (110 zoospores/ml) resulted in 36% and 12% infection rates after 1 and 3 days posttreatment, respectively, and no infections at 6 and 12 days posttreatment.

In the bioassays 3 and 4, batches 1 and 2 of the capsules aged 29–53 days were infective to mosquito larvae. Results were variable due in part to differences in capsule sizes and fungal concentration. A sample from batch 1 stored 53

days gave high larval infections for 24 days after immersion in water, while another sample from the same batch and stored 40 days gave very low infection rates. Samples from batch 2 stored 29 and 40 days were highly infective after 24 days of immersion in water.

In bioassay 5, samples from three batches of capsules, compared after 8, 64 and 75 days storage, were all highly infective to mosquito larvae up to 24 days immersion in water, at which time the assay was terminated. Use of the 8-day-old capsules at different application rates (1–20 capsules per cup) resulted in larval infection rates and residual times proportional to the doses.

Bioassay of encapsulated oospores. Oospore activation and germination was observed in the encapsulated material. Mature oospores (Fig. 3) had a well developed wall and a concentric reserve lipid globule (ooplast) surrounded by a fine-grained cytoplasm as described for viable oospores by Brey (1985) and Kerwin and Washino (1983). Activation and germination of oospores were similar to those described for the same process in the fungi *Phytophthora fragariae* (Duncan 1977) and *Pythium aphanidermatum* (Ruben et al. 1980). Germination resulted in a slight increase in diameter of the oospore and disappearance of the centric ooplast, followed by formation of a germination tube (Fig. 4). Germinating oospores in the capsules were observed throughout the 48 day duration of the bioassays. About 50% of the oospores germinated during the 48-day interval as indicated by microscopic observations of oospores in the capsules.

The bioassay data showed differences in percent infection of larvae exposed to the agar cultures and the capsules prepared and stored in different ways (Table 3). There was a high initial percent infection of larvae exposed to agar culture disks (treatment 1) and a subsequent decline with increasing time after immersion of the agar disks in deionized water. Percent infection of larvae exposed to encapsulated mycelia and oospores held in water (treatment 2) peaked at 12 days postimmersion. Treatments 3 and 4, in which the capsules were frozen to kill the mycelia, leaving only oospores, had highest percent infection at 6 days postimmersion and varied thereafter, up to 48 days postimmersion. Treatments 5 and 6 consisting of encapsulated oospores and mycelia, which were held at room conditions for 15 and 35 days (ca. 25°C), caused 100% initial infection after immersion and a decrease of infection with increasing time postimmersion.

Field trials. The encapsulated *L. giganteum* mycelia (presporangia) were infective to mosquito larvae in outdoor pools. In the first trial (Table 4), there was a gradual decrease in larval abundance in the treated pools during days 2–7 posttreatment and in pupal abundance 3–7 days posttreatment. The treatment rates were high and, consequently, there was no relationship between dose and percent control. At the time of fungal application, the temperature was 29.5°C, dissolved oxygen concentration was 4.4 mg/liter, conductivity ranged from 40 to 100 μmhos/cm and pH varied from 8.1 to 8.5.

In the second trial (Table 5), high levels of

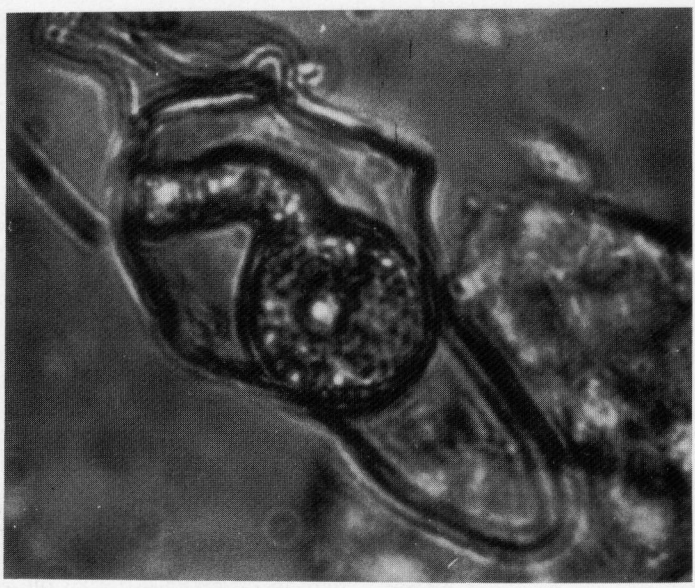

Fig. 4. Germinating oospore of *Lagenidium giganteum* showing formation of a germination tube.

Table 3. Percent infection of *Culex quinquefasciatus* larvae exposed individually to agar culture disks or calcium alginate capsules containing *Lagenidium giganteum* mycelia, oospores, or both, after various periods postimmersion in deionized water.

Treatment	Fungal stages present	Percent larvae infection at days postimmersion					
		1	3	6	12	24	48
1. SFE agar disk	mycelia	100.0	80.4	72.1	25.0	2.5	0.3
2. Encapsulated & held in water 5 days at 23–26°C	oospores and mycelia	9.6	79.2	88.8	95.8	77.9	44.6
3. Encapsulated, held 7 days at 23–26°C & frozen for 8 days	oospores	0	0	100.0	47.1	66.7	80.4
4. Encapsulated, held 7 days at 23–26°C & frozen for 10 days	oospores	4.2	91.7	98.3	94.6	82.1	16.7
5. Encapsulated, held 15 days at 23–26°C	oospores and mycelia	100.0	98.8	100.0	47.1	23.8	2.9
6. Encapsulated, held 35 days at 23–26°C	oospores and mycelia	100.0	94.6	90.4	97.1	81.3	65.4

Table 4. Mean number larvae and pupae and percent control of *Culex quinquefasciatus* in outdoor pools treated with various dosages of *Lagenidium giganteum* mycelia (presporangia) in calcium alginate capsules. Treated June 30, 1986.

Stage	Dose (capsules/pool)	No. per dip and (percent control) at days posttreatment[1]				
		0	2	3	4	7
larvae	1,780	34.2	23.9 (27)	7.5 (72)	0 (100)	0 (100)
	890	30.4	19.0 (42)	4.4 (84)	0.6 (98)	0 (100)
	440	34.5	25.7 (22)	11.9 (56)	5.1 (88)	0 (100)
	220	35.4	26.6 (19)	3.3 (88)	0.1 (98)	0 (100)
	untreated	26.3	33.0	27.1	40.8	44.1
pupae	1,780	1.3	4.7 (0)	0.3 (91)	0.1 (98)	0 (100)
	890	1.8	4.4 (0)	0.7 (79)	2.3 (54)	0 (100)
	440	2.0	4.0 (0)	1.1 (67)	3.5 (30)	0 (100)
	220	0.6	4.5 (0)	0.6 (82)	0.3 (94)	0 (100)
	untreated	2.3	3.5	3.3	5.0	8.0

[1] Values are means from 2 pools (1 pool for untreated) per treatment; 3 dips per pool on each sampling date. Percent control calculated in comparison to the untreated pool.

mosquito control were obtained using capsules of higher calcium alginate concentration and containing either mycelia (presporangia) or oospores. With capsules containing presporangia, 100% larval control was achieved at 6 to 11 days posttreatment with all treatments. The treatment with capsules containing oospores yielded 100% control at 11 days posttreatment but less control than the sporangial treatments at earlier periods posttreatment. There were no consistent differences in the degree of mosquito control with different concentrations of calcium alginate. The capsules containing sporangia had been stored for 16 days at 15°C prior to use while those containing oospores had been stored frozen (−15°C) for 14 days. As noted previously, *Culex* egg rafts were added daily to these screened pools. Also, ca. 20 infected cadavers were removed daily from each pool and infection by *L. giganteum* confirmed microscopically. Water quality was similar to the first trial and water temperature at time of treatment was ca. 25°C.

DISCUSSION

Production of calcium alginate capsules containing *L. giganteum* can be done with simple equipment. The capsules in the present experiment were produced under clean but non-sterile conditions and low level contamination by fungi and bacteria developed during storage; however, the presence of these contaminants did not seem to appreciably affect the infectivity or viability of the encapsulated *L. giganteum*. The fungus was washed 3 times in deionized water prior to encapsulation, eliminating most nutrients in the medium which probably limited the level of contamination by other organisms. Bacterial contamination might be reduced by using an appropriate amount of antibiotics in the medium (Lim and Moss 1981), manipulating the concentration of sodium alginate and $CaCl_2$ during production (Bashan 1986), or by the degree of drying during storage (Mugnier and Jung 1985).

The concentration of presporangia in the capsules in these experiments was not controlled

Table 5. Mean number of larvae and pupae and percent control of *Culex quinquefasciatus* in outdoor pools treated with *Lagenidium giganteum* mycelia (presporangia) or oospores encapsulated in calcium alginate using various concentrations (g/liter) of sodium alginate. Treated August 21, 1986, 200 capsules per pool.

Stage	Treatment and type of capsule	No. per dip and (percent control) after days posttreatment[1]					
		0	2	4	6	9	11
larvae	20 g/l alginate (+ presporangia)	28.6	25.4 (0)	25.7 (46)	0.4 (99)	0 (100)	0 (100)
	16 g/l alginate (+ presporangia)	39.0	30.1 (0)	0.4 (99)	0 (100)	0 (100)	0 (100)
	8 g/l alginate (+ presporangia)	21.6	31.9 (0)	25.4 (46)	0 (100)	0 (100)	0 (100)
	8 g/l alginate (+ oospores)	25.5	33.5 (0)	42.3 (11)	17.4 (49)	7.9 (80)	0 (100)
	untreated	17.4	21.0	47.3	34.3	39.3	27.7
pupae	20 g/l alginate (+ presporangia)	3.3	14.2 (0)	0.7 (59)	3.8 (24)	0 (100)	0 (100)
	16 g/l aginate (+ presporangia)	3.3	13.4 (0)	1.9 (0)	0 (100)	0 (100)	0 (100)
	8 g/l alginate (+ presporangia)	0.5	9.7 (24)	4.5 (0)	0.2 (96)	0 (100)	0 (100)
	8 g/l alginate (+ oospores)	2.4	14.8 (0)	3.0 (0)	1.5 (70)	0.7 (87)	0 (100)
	untreated	3.9	12.7	1.7	5.0	5.3	15.0

[1] Values are means of 2 pools (1 pool for untreated) per treatment; 3 dips per pool on each sampling date. Percent control calculated in comparison to the untreated pool.

between the batches of capsules produced and that likely caused variability in the results of the bioassays. Larval infection rates were higher in bioassays 3, 4 and 5 with capsules from batch 2 (average of 929 presporangia/capsule) than with capsules from batch 1 (325 presporangia/capsule). In the first bioassay, the estimated dose of zoospores was about the same for the capsule and agar-disk treatments (975 and 891 zoospores per ml, respectively), and higher for the treatment with free presporangia (3,905 zoospores/ml). Nevertheless, the encapsulated *L. giganteum* formulation was more persistent than the agar culture and free presporangia material.

Of interest was the capacity of the encapsulated presporangia to remain infective after storage at 15°C for up to 75 days. McCray et al. (1973) reported persistence of infectivity by *L. giganteum* (North Carolina isolate) in mosquito larvae held in water for 5 to 7 days at 26°C and for 14 days when held at 15.6°C. Dried, infected cadavers lost their infectivity as did cadavers on moist filter paper. Jaronski et al. (1983) reported decreased infectivity of 6-day old hemp seed agar cultures of the North Carolina isolate stored at 25°C and 1-week-old cultures stored at 4°C. More recently, Su et al. (1986) reported a ST_{50} (storage time after which there was 50% reduction in infectivity) of 48–49 days of SFE agar cultures (California isolate) stored at 15°C. High infection rates of larvae exposed to 75-day-old calcium alginate capsules in the present bioassays suggest the ability of the presporangia to survive and remain ineffective for long periods of time. Encapsulated presporangia also exhibited prolonged zoosporogenesis, being infective up to 24 days after immersion in water.

The laboratory bioassays with encapsulated oospores of *L. giganteum* showed that these oospores survived the encapsulation process and remained viable after encapsulation and storage (up to 35 days tested). Prolonged storage (several years) of dried oospores has been reported by Kerwin et al. (1986) although germination is variable and unpredictable. Very little data exist to evaluate quantitatively the effect of various factors (temperature, moisture, etc.) on oospore survival and germination rates. In our experiments oospore germination occurred spontaneously under several conditions and drying was not required. Germination of oospores was observed microscopically in capsules that had been immersed in water continuously after their production (Treatment 2, Table 3). Larvae exposed to these capsules became infected throughout the 48-day immersion period, demonstrating fungal activity. Infection rates in treatments 2, 5 and 6 (Table 3), however, could be attributed to either oospore germination or zoosporogene-

sis from remnant mycelia in the capsules. Nonsterility in the bioassays, as well as the addition of liver powder solution to individual cells, may have induced conditions for saprophytic development of *L. giganteum* with subsequent production of some zoospores. However, infection rates in treatments 3 and 4 (Table 3) were the result of oospore germination, since mycelia in the capsules were killed by freezing for 8–10 days. Other bioassays using frozen encapsulated mycelia showed no infection of larvae.

Activation and germination of oospores appears to be a much slower process than the development of presporangia to sporangia which produce vesicles and release zoospores. In treatments with encapsulated oospores only, peak larval infection occurred at 12 days posttreatment, while peak infection was at 1 day posttreatment using capsules containing either presporangia or mycelia and oospores.

In the field trials, there was no effect of dose on the rate of mortality of *Cx. quinquefasciatus* larvae as rather high numbers of capsules per pool were used. Even initial low rates of zoosporogenesis from the capsules could have induced subsequent epizootics. Various samples of water from the experimental pools were returned to the laboratory on the day of treatment and 10–20 capsules were immersed in these samples. Microscopic inspection of these capsules 1–4 days postimmersion revealed reduced vesicle formation and zoosporogenesis (compared to capsules in deionized water) although there was extensive outgrowth of discharge tubes from the capsules. Larvae that were placed in these water samples became infected indicating that there was apparently some zoospore production from the capsules. Numerous capsules were then (after 4 days in the pool water) washed with deionized water and subsequently immersed in deionized water. High numbers of vesicles and zoospores were produced 1–3 days postimmersion in the deionized water, indicating that the sporangia in the capsules were still viable for zoospore production even after 4 days of exposure in pool water. These observations suggest that the rate of zoospore formation by *L. giganteum* in calcium alginate may be affected by water quality as previously documented for unformulated material (Jaronski and Axtell 1982, Lord and Roberts 1985). Depending on water conditions, zoosporogenesis from encapsulated presporangia could be greatly prolonged in a mosquito breeding habitat.

These experiments demonstrated the ability of the asexual and sexual stages of *L. giganteum* to survive the encapsulation process and remain viable in calcium alginate beads. Furthermore, the residual life of the mycelia in the capsules was enhanced in comparison to previously used types of inoculum (agar cultures and zoospores). Encapsulation of *L. giganteum* mycelia in calcium alginate should be an effective method for storing and applying the fungus. The calcium alginate matrix prolongs storage life and provides protection from mechanical abrasion during transportation and application. The use of the encapsulated asexual stages of *L. giganteum* may be more advantageous than using the sexual stage (oospore) without encapsulation since germination of dried oospores of *L. giganteum* has been variable and unpredictable (Kerwin et al. 1986). Even though germination is asynchronous, dried oospores are a promising method of application for long term mosquito control due to the ease of handling and storage. Encapsulation of oospores would be an extra expense and inconvenience but offers the possibility of enhanced activation and germination. Oospores encapsulated in calcium alginate readily germinated after immersion in water in our lab assays and field trial.

Further studies are necessary to evaluate the effectiveness of the encapsulated *L. giganteum* under various field conditions. Modifications and improvements in the encapsulation and storage techniques should enchance the potential for use of *L. giganteum* for mosquito control.

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