COMPARISON OF FLOATING AND SINKING ENCAPSULATED FORMULATIONS OF THE FUNGUS *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES) FOR CONTROL OF *ANOPHELES* LARVAE

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ABSTRACT. Floating calcium alginate capsules containing *Lagenidium giganteum* and 1% ground cork gave higher levels of control of *Anopheles quadrimaculatus* larvae in a 100-cm column of water than sinking capsules containing no cork. There was no significant difference between the cork capsules and the sinking capsules in the infection of larvae by the encapsulated fungus after storage (15°C) for 57 days, although infectivity declined during that time from an initial infection rate of 100% to 35% and 40% for the cork and sinking capsules, respectively. Floating capsules containing glass bubbles were less effective than the cork capsules in the 100-cm column of water and had a shorter storage life than either sinking capsules or cork capsules.

INTRODUCTION

The fungus *Lagenidium giganteum* Couch is a potentially useful biological control agent against mosquito larvae (Jaronski and Axtell 1983, 1984; Lacey and Undeen 1986, Guzman and Axtell 1987a, 1987b; Kerwin and Washino 1988, Lacey and Lacey 1990). Encapsulation of the fungus in calcium alginate beads provides greater retention of infectivity against mosquito larvae and more convenience in storing, handling and application than the direct use of fungus from agar or liquid cultures (Axtell and Guzman 1987, Patel et al. 1990); however, the capsules rapidly sink in water and floating capsules might be more effective, especially against surface-feeding *Anopheles* larvae. Floating and sinking formulations of alginate-encapsulated *L. giganteum* were evaluated for their effectiveness against larvae of *Anopheles quadrimaculatus* Say.

MATERIALS AND METHODS

Mosquito larvae were hatched from eggs provided from a colony of *An. quadrimaculatus* maintained by the U.S. Department of Agriculture, Medical and Veterinary Entomology Laboratory, Gainesville, FL. The *L. giganteum* was routinely maintained in our laboratory in liquid SFE (sunflower seed extract) medium as previously described (Jaronski and Axtell 1984, Guzman and Axtell 1986). The culture was originally obtained from J. L. Kerwin (University of California, Davis, CA) and is referred to as the California isolate. Prior to the encapsulation experiments, the fungus was passed 23 times through larvae of *Culex quinquefasciatus* Say and maintained in liquid SFE medium for ca. 6 months.

Encapsulation: The basic techniques for culturing and encapsulation in calcium alginate were described by Axtell and Guzman (1987a, 1987b) and Patel et al. (1990). Fungus from 8-day-old liquid SFE cultures (100 ml) was encapsulated after microscopic examination to assure that most of the fungus was in the presporangial stage of the asexual cycle. The fungus was allowed to settle to the bottom of the flask, the liquid decanted, the fungus rinsed 4 times with sterile deionized water, reconstituted to 100 ml, blended for 5 sec in an electric blender and mixed with 400 ml of sodium alginate solution (7.5 g of KELGIN HV, Kelco Division, Merck and Co., San Diego, CA) in the blender. Additives to produce floating formulations were added at this point in the procedure. The fungus-alginate mixture was transferred to a glass reservoir containing a magnetic stirrer and placed on a stir plate. The stirred mixture was dripped into a calcium chloride solution (37.5 g/liter) through a series of capillary tubes inserted into a flexible plastic tube attached to the reservoir. The capillary tubes were 1.8 mm diam for all additives except cork, which required 4 mm diam tubes because of the larger particle size. The capsules formed as the sodium alginate precipitated as calcium alginate in the solution. The capsules were removed, washed and stored in closed plastic containers at 15°C.

In preliminary tests, several materials were added at various concentrations (3–5 each) and the resulting capsules added to water and observed to determine the approximate time they would float. The materials and concentrations (%) in the alginate-fungus mixture were: cork (0.1, 0.3, 0.5, 1.0), glass bubbles (0.25, 0.4, 0.5, 1.0, 1.5), sunflower oil (10, 15, 20), motor oil (2, 6, 10), vermiculite (0.25, 0.5, 1.0), styrofoam (0.01, 0.02, 0.1, 0.2), styrene/divinyl benzene

polymer (Supelpak®, Supelco, Bellefonte, PA) (0.1, 0.2, 0.7, 0.9), corn oil (2, 5, 10, 15) and cottonseed oil (2, 5, 10, 15). The capsules with 15 and 20% sunflower oil floated for more than 6 days, but the mixture was too viscous for practical use in encapsulation. Capsules with 1% cork and all concentrations of glass bubbles floated for 6 days or more. Other additives and concentrations yielded capsules that floated for less than 1 day.

Only cork and glass bubbles were used in the floating capsules for the experiments because they remained afloat for the longest time and presented no problems due to high viscosity. The natural cork was a ground and sieved product (No. 14–30) from Maryland Cork Co., Elkton, MD. The irregular particles had maximum widths of 0.4–2.2 mm. The glass bubbles (Scotchlite®, No. C15/250, 3M Co., St. Paul, MN) was a fine powder composed of round particles (0.01–0.02 mm diam). The glass bubbles are commercially used in reflective signs. Five grams of either cork or glass bubbles were mixed with the regular alginate solution to produce the floating capsules containing 1% additive. The capsules produced from the alginate solution with no additive were sinking capsules.

The diameters and mean numbers of presporangia of *L. giganteum* in the capsules were estimated by microscopic examination of 5 representative capsules of each type (Axtell and Guzman 1987). Because it was difficult to microscopically observe the presporangia in the glass and cork preparations, their numbers of presporangia were estimated by calculations using the diameters and the corresponding numbers of presporangia in the sinking alginate capsules reduced by the proportion of glass or cork in the floating capsules. The mean diameter of the sinking capsules was 4.0 ± 0.4 mm and the mean number of sporangia per capsule was 1,015. The mean diameter and the mean number of sporangia for glass capsules were 5.0 ± 0.6 mm and 1,862, respectively. For cork capsules, the mean diameter and mean number of sporangia were 6.2 ± 0.3 and 3,161, respectively.

Efficacy: Capsules were evaluated for the control of *An. quadrimaculatus* larvae in tall opaque PVC (polyvinyl chloride) plastic cylinders (100 cm high, 10 cm diam) containing 8 liters of deionized water and sand in the bottom and held at 26°C. The same batch of each type of capsules were assayed 3 times (after 3, 9 and 17 days storage at 15°C). In these assays 20 sinking, 20 glass or 10 cork capsules were added to each cylinder. The lesser number of cork capsules was to compensate for those being larger and having more presporangia than the other types of capsules.

Microscopic examination of samples of both sinking and floating capsules held in separate containers of water indicated that 2 days' immersion was required for abundant vesicles and zoospores to be produced. Sinking capsules were added to each cylinder and 2 days later mosquito larvae were added. Cork or glass capsules were held for the 2 days in a separate container of water and then added to the cylinders 1 h after adding the mosquito larvae. This procedure was used to assure that zoospores from the floating capsules were not present near the surface of the water at the time the larvae were added. The sinking capsules were added to the cylinders without prior holding in separate containers to assure that zoospores were not poured into the upper levels of the water in the cylinders.

There were 6 cylinders for each type of capsule and for the control (no capsules) and 20 second-instar larvae were added to each cylinder. At the time the larvae were added, 20 ml of a 1:1 yeast/liver powder slurry was added to each cylinder. Dead larvae on the surface were removed every 12 h and microscopically inspected for fungal infection. Removal of floating dead larvae prevented the introduction of additional zoospores at the surface due to infected cadavers. Dead larvae, if any, on the bottom could not be removed because the bottom was difficult to see and any attempt to remove larvae from the bottom would have disturbed the column of water and, consequently, the distribution of zoospores in the column. Also, for those reasons the efficacy of the treatments could not be determined by counts of infected larvae.

The efficacies of the treatments were measured by counts of the numbers of live larvae observed in the top 10 cm of water in each cylinder at 24-h intervals for 3 days. Three counts were made at 15-min intervals and the maximum count for each cylinder used to calculate the mean maximum number of larvae per treatment per day. The data were analyzed by analysis of variance and means compared by Tukey's HSD test to determine significant differences ($P \leq 0.05$) using SAS procedures (PROC ANOVA, PROC MEANS/TUKEY [SAS Institute 1985]).

In the design of the experiment, the validity of the assumption that zoospores from sinking capsules would have limited distribution in a 100-cm column of water was verified by placing capsules in the bottom of cylinders containing a 10, 15, 25, 50 or 100-cm column of water. Sentinel larvae were confined in a floating screen cage on the surface of the water column 2 days

after addition of the capsules. This was repeated 3 times and, in all cases, larvae were infected in water depths of 10, 15 and 25 cm but not in water depths of 50 and 100 cm.

Also, the assumption that the *Anopheles* larvae would distribute unevenly, with most near the surface, in a 100-cm column of water was verified by observing larvae in transparent plastic cylinders. There were 3 cylinders for each instar (second and third) with 50 larvae per cylinder and the experiment was repeated 3 times. The numbers of larvae at increments of 5 cm were counted 3 times at 15-min intervals and the maximum count used. After 1 h of adaptation to the cylinders, about 50% of the second instars and 70% of the third instars were in the top 5 cm of the water column.

Storage: To determine the infectivity of the encapsulated fungus after storing at 15°C for up to 72 days, the capsules were bioassayed using *An. quadrimaculatus* second instar larvae in 100 ml of deionized water in plastic bowls held at 27°C. Capsules (20 sinking, 20 glass or 10 cork) were added to each bowl 2 days prior to adding 20 larvae. There were 4 bowls for each type of capsule and for the control (no capsules). Dead larvae were counted and removed at 12-h intervals for 3 days and microscopically inspected for fungal infection. The data on percentage larval infection were transformed by arcsine $\sqrt{x}$ and analyzed by analysis of variance and means compared by Tukey's HSD test to determine significant differences ($P \leq 0.05$) using SAS procedures (PROC ANOVA, PROC MEANS/ TUKEY [SAS Institute 1985]).

Table 1. Numbers in live *Anopheles quadrimaculatus* larvae in a 100-cm column of water in plastic cylinders treated with *Lagenidium giganteum* encapsulated in calcium alginate with or without glass bubbles or cork.

Treatment	Mean maximum number larvae per cylinder at days after treatment[1]			
	0	1	2	3
Bioassay 1—Capsules stored 3 days				
Alginate	10.5a	16.8a	13.7b	13.0a
Alginate + glass	9.8a	16.5a	11.7b	9.0b
Alginate + cork	9.2a	11.7b	7.2c	6.5b
Control	10.3a	17.8a	17.0a	16.0a
Bioassay 2—Capsules stored 9 days				
Alginate	8.7a	18.3a	16.3a	10.8b
Alginate + glass	8.5a	16.2a	11.2b	6.0c
Alginate + cork	8.2a	9.0b	2.7c	0.8d
Control	7.5a	18.8a	18.7a	14.8a
Bioassay 3—Capsules stored 17 days				
Alginate	12.8a	18.8a	15.0ab	9.8a
Alginate + glass	11.5a	19.2a	15.0ab	10.8a
Alginate + cork	13.5a	16.7b	10.3b	7.3a
Control	13.5a	19.0a	17.0a	13.8a

[1] Means with same letter in a column within a bioassay were not significantly different ($P \geq 0.05$, Tukey's HSD, [SAS 1985]).

RESULTS AND DISCUSSION

Overall, the cork capsules were more effective in controlling the *Anopheles* larvae than were the glass capsules or the sinking capsules (Table 1). Within each assay of the 3 age groups of capsules, there were no significant differences in the numbers of larvae initially (day 0). At 1, 2 and 3 days after treatment with cork capsules stored for 3, 9 or 17 days at 15°C, the cylinders with capsules had significantly fewer larvae than the untreated control, except for the bioassay of 17-day-old capsules at 3 days after treatment. At 1 day after treatment, the glass and sinking capsules stored 3, 9 or 17 days had no significant effect on the numbers of larvae. At 2 days after treatment, the cylinders with 3-day-old glass or sinking capsules and those with 9-day-old glass capsules had significantly fewer larvae than the control, but both types of capsules were significantly less effective than the cork capsules. At 3 days after treatment, the 3- and 9-day-old glass capsules gave significant reductions in the numbers of larvae but significantly less than produced by the cork capsules only for the 9-day-old capsules. The 3- and 9-day-old sinking capsules at 3 days after treatment were less effective than the glass or cork capsules. The 17-day-old glass and sinking capsules did not significantly reduce the numbers of larvae at 1, 2 or 3 days after treatment.

In the first bioassay, 93% of the total number of dead larvae examined ($n = 28$) from cylinders with sinking capsules were infected, 96 and 100% from those with glass capsules ($n = 49$) or cork capsules ($n = 60$), respectively. In the second bioassay, 90% of the dead larvae examined ($n = 20$) from cylinders with sinking capsules were infected, 97 and 100% from cylinders with glass capsules ($n = 38$) or cork capsules ($n = 79$), respectively. In the third bioassay, 100% of the dead larvae retrieved from cylinders with sinking capsules ($n = 4$) or cork capsules ($n = 36$) were infected, 93% from cylinders with glass capsules ($n = 14$).

In the bioassay of the encapsulated fungus after various storage periods, the sinking capsules and cork capsules produced 100% infection of the larvae after storage for 3–23 days (Table

Table 2. Mean percent infection[1] of *Anopheles quadrimaculatus* larvae by *Lagenidium giganteum* encapsulated in calcium alginate with or without glass bubbles or cork and bioassayed after various storage periods at 15°C.

Type of capsule	Infection after storage time (days)							
	3	9	17	23	30	44	57	72
Alginate	100	100a	100a	100a	46.7a	43.8a	40.0a	0.0a
Alginate + glass	100	92.5b	61.3b	30.0b	26.7a	0.0b	0.0b	0.0a
Alginate + cork	100	100a	100a	100a	42.5a	41.3a	35.0a	0.0a

[1] Means with same letter in a column were not significantly different ($P \geq 0.05$, Tukey's HSD, [SAS 1985] on data transformed by arcsine $\sqrt{x}$). No dead infected larvae were found in the control (no capsules) at any time period.

2). After storage of sinking and cork capsules for 30–57 days, there were similar decreases in larval infection and no infection after storage for 72 days. There were no significant differences between the cork capsules and sinking capsules with respect to percentage infection of larvae following capsule storage for 3–72 days. Glass capsules, after storage for 3 days produced 100% infection; there was a decrease in infection after storage for 9–30 days and no infection after storage for 44 days. The percentages of larvae infected with the fungus encapsulated in glass capsules were significantly lower than those infected by sinking capsules or cork capsules following capsule storage from 9 to 23 days.

The low numbers of live larvae in the cylinders with floating cork or glass capsules indicated that the infectivity of the fungus in the floating formulations was high with probably high densities of zoospores in the region of greatest larval abundance, i.e., the top 5 cm of water in a cylinder. However, the zoospore densities were apparently not high enough to kill all of the larvae and probably not as high as the densities in the small bowls used to bioassay storage effects (Table 2). Lower densities of zoospores near the surface in the cylinders was likely because of the large volume of water and the opportunity for the zoospores to move downward in the column.

The infectivity of the fungus in the cork and sinking capsules in the small volume of water in the bowls remained similar for all storage intervals studied. The more rapid decline in the infectivity of the fungus encapsulated with glass bubbles may have been caused by broken particles that we observed in the glass bubble powder. Perhaps the broken particles caused abrasion of the presporangia and hampered formation of exit tubes required for the production of vesicles and zoospores by the encapsulated fungus.

Based on these data, *L. giganteum* encapsulated in calcium alginate containing 1% (w/w) ground cork particles (0.4–2.2 mm maximum dimensions) float in water and yield higher levels of control of surface-feeding *Anopheles* larvae than sinking capsules containing no additive.

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