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COMPARISONS OF DIFFERENT TYPES AND CONCENTRATIONS OF ALGINATES FOR ENCAPSULATION OF *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES), A FUNGAL PATHOGEN OF MOSQUITO LARVAE

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ABSTRACT. Six different types of alginates used to encapsulate *Lagenidium giganteum* gave similar levels of fungal infection in *Culex quinquefasciatus* larvae. Initial infection levels when the capsules were immersed in water after 6 days of storage (15 and 25°C) were 100% for all types of alginate and after 42 days of storage was 62–100%, depending on the type of alginate. Infectivity was 24–100% after the encapsulated fungus were left in water for 7 days and after 15 days was 0 to 26%, depending on the alginate. When 2 of the alginates were tested at different concentrations to give high, medium and low viscosity solutions, the fungus encapsulated using lower concentration alginate solutions usually gave the highest level of infectivity.

INTRODUCTION

The fungal pathogen *Lagenidium giganteum* Couch is a promising biological control agent of mosquito larvae (Jaronski and Axtell 1983, Lacey and Undeen 1986, Kerwin and Washino 1988). Axtell and Guzman (1987) first reported on the encapsulation of the fungus in calcium alginate beads. The encapsulated presporangial mycelia remained infective to mosquito larvae after long periods of storage (up to 75 days at 15°C) and after prolonged immersion in water (up to 24 days). Encapsulation has the added advantage of offering greater convenience in storing, handling and application. We evaluated the suitability of several commercial types of alginate in addition to the one used by Axtell and Guzman (1987) and determined the effect of viscosity of the alginate on the survival and infectivity of the encapsulated fungus.

MATERIALS AND METHODS

Fungus cultures: The California isolate (obtained in 1987 from J. Kerwin, University of California, Davis, CA) was used and maintained in liquid culture in a water extract of sunflower seeds (SFE) as previously described (Jaronski and Axtell 1984, Guzman and Axtell 1986). Fungus from 10-day-old cultures grown on liquid SFE was encapsulated after microscopic examination to assure that the cultures were almost entirely in the presporangial stage and no oospores were present.

Alginate solutions: The tradenames of the 6 alginates (and lot numbers), obtained from Kelco Division, Merck & Co., San Diego, CA, were KELGIN LV (38028A), KELGIN HV (31524A), KELTONE LV (35245A), KELTONE HV (33833A), MANUGEL DMB (501271) and KELMAR (37168A). These alginates differed in their physical and chemical characteristics (Table 1) according to data from Kelco (Anonymous 1987) and the flow rates, which we determined by timing the flow of 200 ml of each alginate through a funnel with a 4 mm diam opening at the bottom. All were sodium alginates except for KELMAR, a potassium alginate. The Fisher brand unrefined granular sodium alginate (lot no. 853628), previously used for encapsulation studies in our laboratory (Guzman and Axtell 1987), was included for comparison; but its properties were not documented as the product is no longer available. We estimated its viscosity to be ca. 3,000 centipoises (cps) as a 2% solution.

In the first experiment, 8 g of each alginate was blended, at high speeds in a commercial blender, into 400 ml of sterile deionized water (ca. 2% solution). In the second experiment, different concentrations of KELGIN LV and KELGIN HV were prepared to obtain solutions with estimated viscosities of 500, 5,000 and 10,000 cps based on data provided by Kelco (Anonymous 1987) and by measuring the flow rates.

Encapsulation procedure: The procedure used for encapsulating the fungus in the prepared alginate solutions was described by Axtell and Guzman (1987). The fungus in a 100 ml liquid SFE culture (10-day-old) was rinsed 3 times with sterile distilled water, reconstituted to 100 ml, and blended for 5 sec at high speed in a commercial blender. It was then mixed with 400 ml of an alginate solution held in a reservoir containing a magnetic stirrer and placed on top of a stir plate. The stirred alginate and fungus mixture was dripped into a calcium chloride solution (36.8 g/liter $CaCl_2 \cdot 2H_2O$) through capillary tubes (1.6–1.8 mm diam) inserted into a flexible plastic tube attached to the reservoir. Capsules were formed as the alginate precipi-

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Table 1. List of alginates (trade names) and their selected characteristics.

Type and name	Form	Mesh	Viscosity[1] (cps) 1%	Viscosity[1] (cps) 2%	Flow rate[2] (ml/min)	Typical % Na	Typical % Ca
Refined sodium alginates							
KELGIN LV	Granular	40	60	500	50.0	8.4	1.2
KELGIN HV	Granular	30	800	10,000	1.6	8.4	1.2
Gelling grade sodium alginates							
KELTONE LV	Fibrous	150	50	250	67.0	9.4	0.2
KELTONE HV	Fibrous	80	400	3,500	4.2	9.4	0.2
MANUGEL DMB	Granular	150	300	2,000	9.1	9.6	0.2
Refined potassium alginate							
KELMAR	Granular	100	270	3,200	6.3	0.4	1.2

[1] Viscosity measured by the Brookfield Synchr-Lectric Viscometer (Brookfield Engineering Laboratories, Stoughton, MA 02072) Model LVF running at 60 rpm with appropriate spindle at 25°C.

[2] 2% solutions.

tated as calcium alginate in the calcium chloride solution. The capsules were removed, washed and stored in closed containers at 15 and 25°C. The first experiment was repeated with 2 batches of capsules made about a month apart. One batch of capsules was made for the second experiment.

Assay procedure: About 100 capsules from each batch, at each storage temperature, and from each alginate preparation were removed after storage for different time intervals for bioassays. Twenty capsules were placed in each of four 240-ml cups containing 100 ml of deionized water and held at 27°C. Twenty 2- to 3-day-old larvae (second or early third instar) of *Culex quinquefasciatus* Say from a laboratory colony were added to each cup at various intervals after immersion of the capsules in water. At the time the larvae were added, 0.25 ml of a liver powder slurry (35 mg/ml) was added to each cup. Subsequently, dead larvae were counted, removed and checked for infection every 12 h for 3 days. This removal of the dead larvae twice daily prevented recycling of the fungus. These assays were conducted at 1, 4, 7 and 15 days after immersion of the capsules in water in the first experiment and at 1, 7 and 15 days in the second experiment. In both experiments, the assays were conducted after the capsules had been stored at 15 and/or 25°C for various periods up to 42 days.

For each batch of capsules, the mean number of *L. giganteum* presporangia per capsule and mean capsule diameter were estimated from microscopic examination of flattened capsules as described by Axtell and Guzman (1987). Arcsine transformations of the percent infected larvae were used in analysis of variance tests with SAS PROC ANOVA (SAS Institute 1982), and means for different treatments were compared using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Freshly made capsules varied in their appearance and ranked from the most transparent to the most opaque as follows: MANUGEL DMB, KELTONE LV, KELTONE HV, Fisher, KELMAR, KELGIN LV and KELGIN HV. Intermediate flow rates (Table 1) allowed for capsules to be prepared in a reasonable amount of time and resulted in round capsules. With high flow rates, the capsules formed quickly but in clumps if the calcium chloride solution was not vigorously stirred. With low flow rates, well-rounded beads were formed but too slowly; almost 4 h were required to make capsules from 500 ml of the thickest alginate formulation (KELGIN HV).

The mean diameter of flattened capsules prepared with the 7 alginates ranged from 3.9 to 4.5 mm in the first batch and 4.1 to 4.5 mm in the second batch, while the mean number of presporangia per capsule ranged from 2,560 to 3,749 and 2,328 to 4,551, respectively. Capsule size and number of presporangia per capsule were not related consistently to differences in the type and viscosity of the alginates. Since large numbers of presporangia were encapsulated in all cases and each presporangium has the potential of producing about 15 zoospores (Axtell and Guzman 1987), any differences in the infectivity of the encapsulated fungus should not have been due to limited amounts of fungus but rather to differences in the alginates.

There were no significant differences between batches or storage temperatures with respect to percentage infection of larvae exposed to the encapsulated fungus. Therefore, the data were combined for all batches and storage temperature conditions.

In the first experiment, the fungus in all of the alginates assayed after the capsules were in

water for 1 day resulted in 100% infection of larvae except for the fungus in the capsules stored for 42 days, which gave 62–97% infections (Table 2). Differences between alginates were most evident in assays of the capsules after immersion in water for 7 and 15 days. After the capsules were in water for 7 days following storage for 6–42 days, the infectivities of the fungus in the capsules varied greatly among the alginates (23–99%). At 15 days after the capsules were in water, after storage for 13 or 42 days, most of the infectivity was lost in all cases (0–25% infection). All of the alginates seemed to be suitable for encapsulation of *L. giganteum* although KELGIN HV consistently resulted in higher infection rates.

These data confirm the viability of the encapsulated fungus over long periods of storage as reported by Axtell and Guzman (1987). The encapsulated fungus maintained a high level of infectivity to mosquito larvae for 42 days (the maximum duration of capsule storage in this experiment) in contrast to the more rapid loss of infectivity of the fungus stored on SFE-agar as reported by Su et al. (1986). These data also confirm the persistence of the infectivity of the fungus for more than 7 days after the capsules are immersed in water. The infectivity was very low after 15 days of immersion, but low levels of infectivity might be sufficient to induce epizootics under field conditions (Guzman and Axtell 1987).

In the second experiment the capsules prepared with KELGIN LV and KELGIN HV at 3 viscosities ranged in size (diam of flattened capsule) from 3.9 to 5.0 mm while the number of sporangia per capsule ranged from 1,366 to 2,121 (Table 3). The effect of viscosity of the alginate solutions on the percent infection of mosquito larvae by the encapsulated fungus are presented in Table 4. The trend was for reduced infectivity with higher viscosities. After storage for 6 days, the differences were significant for KELGIN LV capsules immersed in water for 15 days and KELGIN HV capsules in water for 7 days. The effects of viscosity were significant for both alginates after 7 days immersion in water following 13 days of storage. After 28 or 42 days of storage, significant differences in the infectivity of the fungus encapsulated in the various concentrations of the two alginates occurred only for the assays done after immersion of capsules in water for 1 day. When the data for each alginate were pooled for all concentrations tested, there were no significant differences in percent infectivity of the fungus in the alginates at any storage or immersion time.

The data suggest that the viscosity of the alginate affects the infectivity of the encapsulated fungus, with the thicker materials tending to give reduced infection of exposed mosquito larvae. However, the more viscous materials produce more rounded and uniform capsules which are convenient for handling and application. A practical compromise would be to select an intermediate viscosity material of around 5,000 cps which would continue to give good infection of exposed mosquitoes at levels close to those obtained with low viscosity materials and yet be convenient for handling and spreading. To obtain a 5,000 cps viscosity solution, 1.6% of KELGIN HV is required. This concentration is half that required for making a KELGIN LV solution with the same viscosity. Fungus encapsulated in formulations of both alginates produced similar infection rates, irrespective of the length of storage time of capsules or the number of days the capsules were kept immersed in water.

Table 2. Mean percent infection of *Culex quinquefasciatus* larvae by *Lagenidium giganteum* mycelia (presporangia) encapsulated in various alginates and bioassayed after the capsules were immersed in water for 1–15 days following storage for 6–42 days.[1]

	Storage time (days)										
	6			13				42			
	Days capsules in water before assay										
Alginate	1	4	7	1	4	7	15	1	4	7	15
KELGIN LV	100	99.7ab	28.9c	100	98.9a	87.4abc	0.0b	97.2a	90.4a	71.1a	15.9a
KELGIN HV	100	100a	99.5a	100	99.9a	99.7a	25.8a	62.0a	54.9a	51.0a	18.5a
KELTONE LV	100	96.9ab	23.8c	100	96.7a	71.1c	0.0b	96.3a	77.8a	28.6a	7.8a
KELTONE HV	100	100a	80.4ab	100	99.9a	98.7a	2.2b	80.4a	81.6a	54.2a	16.7a
MANUGEL DMB	100	93.9b	32.6c	100	99.8a	72.7bc	0.0b	99.7a	64.1a	62.4a	19.1a
KELMAR	100	99.7ab	57.6bc	100	99.1a	88.7abc	2.2b	87.3a	56.3a	39.9a	2.7a
Fisher	100	96.6ab	65.9bc	100	98.9a	97.4ab	7.5ab	65.8a	48.4a	34.7a	14.8a

[1] Each mean is based on 2 batches per storage temperature, 4 replicate cups per batch, 20 larvae per cup. Data for capsules stored at 15 and 25°C were pooled for analysis. Means with same letter in a column were not significantly different ($P > 0.05$, Tukey's test [SAS 1982] using data transformed by arcsine $\sqrt{x}$).

Table 3. Viscosity, concentration (%), mean relative size (diameter) of flattened capsules, and mean no. of presporangia of *Lagenidium giganteum* per capsule for 2 sodium alginates.[1]

Alginate	Viscosity (cps)	Conc. (%)	Mean diameter (mm) of capsule	Mean no. sporangia per capsule
KELGIN LV	500	2.0	3.9a	2,121a
	5,000	3.2	5.0b	1,367a
	10,000	4.0	5.1b	1,712a
KELGIN HV	500	0.9	4.5a	1,366a
	5,000	1.6	4.4a	1,787a
	10,000	2.0	4.5a	1,726a

[1] Means based on 5 capsules from each alginate preparation. Means with the same letter in a column are not significantly different. ($P > 0.05$, Tukey's test [SAS 1982]).

Table 4. Mean percent infection of *Culex quinquefasciatus* larvae by *Lagenidium giganteum* mycelia (presporangia) encapsulated in KELGIN LV and KELGIN HV alginates at three viscosities after the capsules were immersed in water for 1–15 days following storage for 6–42 days.[1]

Viscosity (cps)	Storage time (days): 6			13			28			42		
	Days capsules in water before assay: 1	7	15	1	7	15	1	7	15	1	7	15
KELGIN LV												
500	100	97.5a	26.2a	100	100a	36.6a	100	49.1a	4.4a	98.6a	20.5a	0.0a
5,000	100	66.4a	1.2ab	100	79.8ab	3.4b	100	41.1a	2.5a	88.3a	14.3a	0.0a
10,000	100	80.2a	0.0b	100	69.4b	4.0b	100	13.4a	0.3a	68.2b	10.7a	0.0a
KELGIN HV												
500	100	99.4a	6.5a	100a	100.0a	15.1a	100a	71.6a	1.7a	99.7a	18.9a	2.6a
5,000	100	68.1b	2.1a	100a	96.9a	2.1a	98.8a	65.8a	0.3a	76.1ab	14.9a	0.0a
10,000	100	51.7b	0.0a	99.4a	60.4b	0.3a	52.9b	39.4a	0.0a	31.3b	9.2a	0.0a

[1] Means for 13 and 28 days storage are based on pooled data of capsules stored at 15 and 25°C. Means for 6 and 42 days storage are based on data for 25°C only. Means with same letter in a column were not significantly different ($P > 0.05$, Tukey's test [SAS 1982] on data transformed by arcsine $\sqrt{x}$).

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