FACTORS AFFECTING STORAGE OF MYCELIAL CULTURES OF THE MOSQUITO FUNGAL PATHOGEN *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES)[1]

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ABSTRACT. Sunflower seed extract (SFE) agar cultures (in petri dishes) of *Lagenidium giganteum* (California isolate) were evaluated for zoospore production and ability to infect mosquito larvae, *Culex quinquefasciatus*, after periods of storage up to 93 days at 15°C. Rates of decrease in zoospore production and infectivity were related to soluble protein concentration in the SFE-agar media but at all concentrations (0.7–6.0 mg/ml) about 50% of the initial levels were lost after 40–50 days of storage. Water loss from the SFE-agar did not affect zoospore production or infectivity except at extremely high levels (about 98% water loss).

INTRODUCTION

Jaronski and Axtell (1984) described a procedure for culturing the fungal pathogen of mosquito larvae, *Lagenidium giganteum* Couch (Oomycetes: Lagenidiales), in Sunflower Seed Extract (SFE). Production involves both water dilutions of the SFE (liquid phase) and agar containing SFE (solid phase). The SFE-agar cultures can be blended in water and dispensed into mosquito breeding sites by sprayer or sprinkling can. They reported that agar cultures of the fungus (in 100 mm petri dishes) could be stored for up to 12 wk at 15°C. However, little information was provided on the effects of different storage periods on the ability of the fungus to infect mosquito larvae.

Optimum temperatures for fungal development and infectivity have been reported to be between 20 and 30°C for the North Carolina and Louisiana isolates (Domnas et al. 1982, Jaronski et al. 1983, Jaronski and Axtell 1983). However, very limited quantitative data are available on the effects of prolonged storage on the fungal infectivity. Jaronski et al. (1983), using a North Carolina (NC) isolate of *L. giganteum* plated on hemp seed agar reported loss of zoosporogenesis after 2–3 wk at 25 to 27°C and after 1 wk at 4°C. Guzman and Axtell (1986) observed differences in infectivity of *L. giganteum* cultured in SFE with different concentrations of soluble protein. There are other reports on the effect of culture nutrients on zoosporogenesis of *L. giganteum* (Domnas et al. 1977), but the interaction between nutrient concentration and storage period has not been investigated.

A California (CA) isolate of *L. giganteum* was used to determine the effects of prolonged storage at 10 and 15°C in SFE-agar containing various nutrient concentrations (measured by soluble protein concentration assays). Additional experiments were conducted to determine the effect of water evaporation from the SFE-agar on the fungus infectivity after prolonged storage.

MATERIALS AND METHODS

FUNGAL ISOLATE. The *L. giganteum* isolate used in this study was maintained by weekly mycelial subculturing in SFE liquid media with a soluble protein concentration of 2 mg/ml. This isolate was obtained from J. Lord, Boyce Thompson Institute, Ithaca, NY and originated from J. Kerwin, University of California, Davis. The isolate is referred to as the California (CA) isolate (ATCC, 12301 Parklawn Drive, Rockville, MD, 20852, USA, Accession no. 52675). Protein concentration was determined by the Bradford method (Bio-Rad Chemical Div., Richmond, CA 94804) for all experiments.

VESICLE COUNTS. Circular disks (200 mm^2) were removed from agar culture dishes of each medium after each storage period and individually immersed in 20 ml of deionized water to induce vesicle formation and zoosporogenesis. At 6 hr intervals, the disks were gently removed from the water and observed under a compound microscope at 100X magnification (3.14 mm^2 field of view). Mature vesticles (zoospores well differentiated and close to being released) were counted in five fields of view on each of four disks from each agar dish. The mean number of vesicles per field was determined for each dish and plotted with respect to time after immersion in deionized water. The total number of vesicles produced per field during the entire zoosporogenesis

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interval was estimated by calculating the area under the resultant vesicle-time curve (Guzman and Axtell 1986).

INFECTIVITY ASSAYS. Four circular culture disks (20 mm^2) were removed from each dish as described above and individually immersed in 100 ml of deionized water with 25 3-day-old larvae to induce larval infection by *L. giganteum*. The cups were examined at 6 hr-intervals and dead larvae were removed, observed for infection and discarded. The surviving larvae were fed daily with 0.25 ml liver suspension (35 mg powder/ml).

EXPERIMENTS. For the first experiment, *L. giganteum* was inoculated (1 ml of partially homogenized mycelia from liquid SFE cultures, see Jaronski and Axtell 1984) onto SFE-agar in petri dishes (100 mm diam) containing different concentrations of soluble protein (0.7, 1.5, 3.0 and 6.0 mg/ml). There were 24 dishes per protein concentration. After inoculation, the dishes were held at room temperature (24–27°C) for about 18 hr to allow the fungus to develop evenly over the entire surface of the agar and then were stored at 15°C in closed plastic bags. In addition, 24 dishes (1.5 mg/ml soluble protein) were stored at 10°C in closed plastic bags for 7, 17, 27 and 37 days. Each dish was weighed before and after pouring of the SFE agar and fungal inoculum, and prior to and after the respective storage period. These data were used to calculate the weight lost from each agar culture during the storage period. Three culture dishes of each protein concentration were removed from the 15°C storage after of 7, 17, 27, 37, 47, 57, 67 and 93 days; at each of these times the fungus was assayed for its ability to produce vesicles (structures from which zoospores are released) and infect larvae of *Culex quinquefasciatus* Say.

A second experiment was conducted to determine the effect of water evaporation from agar culture dishes on the infectivity of *L. giganteum* after prolonged storage. The petri dishes were weighed as in the first experiment. The dishes were stored inverted (resting on cover) and normal (resting on bottom) to determine if there were differences in water evaporation due to the position of the plates during storage. The fungus was bioassayed for its infectivity as described above after storage periods of 27, 47 and 67 days at 15°C in media containing 3.0 or 6.0 mg/ml protein concentration. There were eight plates for each period-concentration combination with four agar disks sampled from each dish for zoospore production and infectivity were assayed as described above.

To further determine the effects of water evaporation, agar cultures (3 mg/ml soluble protein concentration) were stored at 15°C for 18 days. These cultures were allowed to dry at varying rates by daily removing water (under sterile conditions) that had condensed on the cover of the dish. Some of the plates were then sealed with adhesive tape to reduce further evaporation. The plates were weighed as described previously. Also, infection rates and number of vesicles were determined by techniques described above.

STATISTICAL ANALYSIS. Analyses were performed using SAS procedures (SAS Institute 1982). The data were analyzed by analysis of variance (PROC ANOVA) to determine overall significant differences between treatments. Specific treatment differences were tested for significance using Duncan's multiple range test (Duncan 1955) at the 0.05 significance level. Linear regression analysis (PROC REG) was conducted to determine quantitative relationships between percent infection or vesicle formation and storage period. Correlation analysis (PROC CANCORR) was conducted to determine the effects of evaporation on infectivity of *L. giganteum*.

RESULTS

Storage period and protein concentration had a significant effect (ANOVA, $P = 0.05$) on the number of vesicles produced and infectivity rates in mosquito larvae by the California isolate of *L. giganteum*. Percent infectivity and number of vesicles decreased with increasing storage period and increased with increasing nutrient concentration in the media (Table 1). Cultures stored at 10°C had reduced infection ability in comparison to cultures stored at 15°C (Table 1). The storage times (at 15°C) after which fungal infectivity decreased by 10, 50 and 90% (ST_{10}, ST_{50} and ST_{90}, respectively) of initial levels tested (7 days storage period) were not significantly influenced by protein concentration (Table 2). Linear regressions between storage period and percent infection, and between storage period and number of vesicles indicated a higher negative slope at higher protein concentrations (Fig. 1). Correlation of percent infection (arcsin transformed values) and number of vesicles per 3.14 mm^2 was significant ($r = 0.88$) suggesting that zoospore infectivity was not affected by storage period or protein concentration. There was no significant correlation between infection rates and weight loss resulting from water evaporation during storage from plates in the first experiment. In addition, there were no significant differences in moisture loss of cultures stored in an inverted versus normal position in the

Table 1. Effect of storage period (at 15° and 10°C) and nutrient concentration (soluble protein) of agar cultures on vesicle formation and infectivity of *L. giganteum* to the mosquito larvae, *Culex quinquefasciatus*.

Temp. °C	Protein conc. mg/ml	Vesicle production and infectivity after given storage periods (days)							
		7	17	27	37	47	57	67	93
		Mean no. of vesicles per 3.14 mm² agar[ab]							
15	0.7	46a	64a	61a	80a	59a	33a	0a	0a
	1.5	163b	99b	82a	94ab	56a	24a	2a	0a
	3.0	283c	196c	194b	104b	70a	40a	67b	4a
	6.0	340d	161d	281c	173c	67a	98b	55b	44b
		Mean percent infection[ac]							
	0.7	15.4a	43.6a	27.4a	24.5a	16.1a	2.0a	0.3a	0a
	1.5	59.0b	66.1b	47.2a	29.6a	24.3a	25.0a	3.4a	0.7a
	3.0	94.6c	79.5c	52.0b	20.5a	15.7a	44.9b	14.4a	16.2b
	6.0	95.9c	85.1d	75.8c	34.8a	28.1a	45.2b	10.7a	18.8b
		Mean no. of vesicles per 3.14 mm² agar[b]							
10	1.5	149	79	1	0	—	—	—	—
		Mean percent infection[c]							
	1.5	86.1	35.9	0.7	0	—	—	—	—

[a] Means followed by the same letter within a column were not significantly different (Duncan's Multiple Range Test, P = 0.05).
[b] Three dishes per concentration per storage period; four agar disks per dish.
[c] Three dishes per concentration per storage period; four cups per dish.

second experiment and therefore the data were pooled (Table 3). Moisture loss increased from a mean value of 11.9% after 27 days storage to 20.8% after 67 days of storage. There was a corresponding decrease in number of vesicles and infection rates with increasing storage period. Within each storage period there were no significant differences in water loss due to protein concentration.

Water evaporation from the agar cultures had a significant effect on infectivity only at the highest level of evaporation achieved (98.4% weight loss) in plates with 3.0 mg/ml protein stored for 18 days at 15°C (Table 4).

DISCUSSION

Sunflower seed extract-agar cultures of *L. giganteum* mycelia produced zoospores which were infective to mosquito larvae at about 50% of the initial production and infectivity levels after about 40 to 49 days of storage at 15°C. This viability after storage was not greatly influenced by the concentration of SFE in the storage media, although higher protein concentration was associated with a higher initial infectivity of the cultures. Water evaporation during storage did not affect infectivity, except at an extremely high level of water loss. Nutrition and metabolism seemed to be the major physiological factors affecting prolonged survival of *L. giganteum* in storage. The decrease in zoosporogenesis after prolonged storage was a result of cellular death in the mycelia. Dead cells were recognized by their characteristic lack of internal cellular structures. Other studies (Domnas et al. 1982) have shown short term inhibition of zoosporogenesis due to nutritional factors and not to fungal mortality. Our data indicate that nutrient

Table 2. Storage times in days at 15°C required for 10, 50 and 90 percent decrease (ST_{10}, ST_{50} and S_{90}) in vesicle production and percent infection of mosquito larvae, *Culex quinquefasciatus*, by *L. giganteum* on SFE-agar (Based on data in Table 1).

Protein conc. mg/ml	Vesicle production			Percent infection		
	ST_{10}	ST_{50}	ST_{90}	ST_{10}	ST_{50}	ST_{90}
0.7	9.2	48.6	87.5	9.3	46.8	84.1
1.5	8.3	39.7	71.2	9.1	45.6	82.1
3.0	8.2	41.5	74.6	8.3	41.5	74.8
6.0	9.7	46.1	83.0	9.4	47.0	84.6

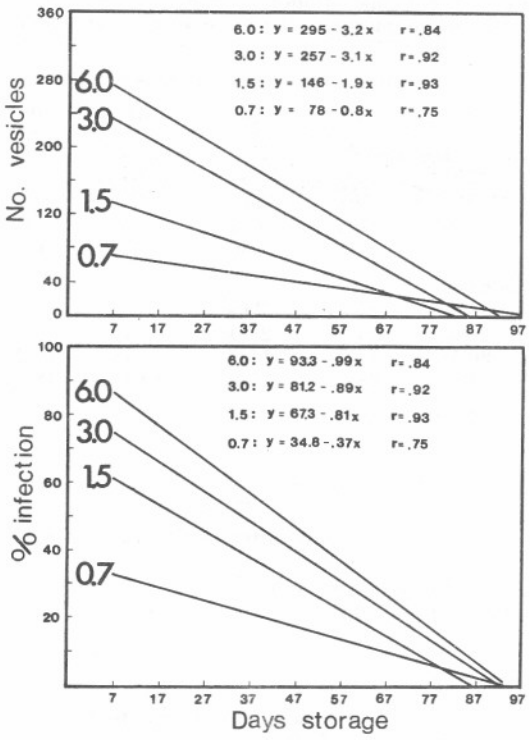

Fig. 1. Linear regressions of percent infection of mosquito larvae, *Culex quinquefasciatus*, and number of vesicles produced by *Lagenidium giganteum* (CA isolate) versus days in storage (15°C) of SFE-agar cultures containing 0.7, 1.5, 3.0 or 6.0 mg soluble protein per ml.

concentration largely affected fungal survival rather than physiological induction of zoosporogenesis. It is likely that nutrient concentration (measured as soluble protein)

Table 3. Percent weight loss, number of vesicles produced per 3.14 mm² agar and percent infection of mosquito larvae, *Culex quinquefasciatus*, by *L. giganteum* produced from SFE-agar cultures in relation to exposure period and protein concentration of the agar.

Period of exposure (days)	Protein conc. (mg/ml)	Mean % weight loss[a]	Mean no. vesicles[a]	Mean % infection[a]
27	3.0	11.7a	305a	63.9a
	6.0	12.0a	190a	65.1a
47	3.0	8.5a	132a	65.6a
	6.0	10.0a	125a	71.6a
67	3.0	19.9a	105a	8.3a
	6.0	21.7a	98a	23.3a

[a] Means followed by the same letter within a column for each storage period, were not significantly different (Duncan's Multiple Range Test, P = 0.05). Each mean based on four samples from each of eight dishes.

Table 4. Effect of evaporation of water from SFE-agar cultures of *L. giganteum* stored at 15°C for 18 days on the vesicle production and infection of mosquito larvae, *Culex quinquefasciatus*.

	Vesicle production	
% weight loss	Mean no. per 3.14 mm² agar[a]	Mean % infection[a]
20.8	240 ± 13a	54.3 ± 18.0a
46.4	275 ± 46a	63.8 ± 8.0a
69.0	236 ± 61a	55.0 ± 3.2a
88.7	225 ± 7a	70.2 ± 3.6a
98.4	41 ± 35b	11.0 ± 12.0b

[a] Means followed by same letter within a column were not significantly different (Duncan's Multiple Range Test, P = 0.05). Each mean based on four agar disk samples from each of five dishes.

increased the rate of fungal development and metabolism with a corresponding increase in enzymatic activity in the medium. The accumulation of extracellular metabolic products may cause changes in the osmotic conditions of the medium or lysis of the cells which would result in fungal mortality after a culture has reached maximal growth (Cochrane 1958).

Although the rate of decrease in infectivity of mosquito larvae by the fungus was higher at the higher protein concentrations, these cultures remained viable for a longer period due to their initial higher infectivity rates relative to the cultures containing lower protein concentrations. The loss of infectivity with increasing storage period needs to be considered in planning application of *L. giganteum* into natural mosquito-breeding habitats. Care must be taken in calculating application dosages using culture material of varying age. Although SFE-agar cultures of *L. giganteum* will retain some viability for longer periods, we recommend that storage at 15°C not exceed 40 to 50 days since after that time period about 50% of the initial infectivity will be lost. For such prolonged storage, the SFE concentration measured as soluble protein by the Bradford method should be 6 mg/ml to assure ample nutrients.

There was no correlation between water loss and reduced virulence of the fungus. The fungus was inhibited only at very high evaporation rates (98%). These data indicate that water evaporation from the plates during storage will not cause detrimental effects on fungus infectivity. Although there is water evaporation, the confined microhabitat in the petri dish is sufficient to maintain close to 100% relative humidity in the medium.

The survival of *L. giganteum* during storage

may vary if other types of media are used. Physiological studies with other fungi have shown that the type of medium in which a particular fungal species is cultured can influence the type of metabolites being produced. These metabolites can accumulate during storage and can create conditions which affect fungal survival to varying extents. Several types of solid media have been used for culturing *L. giganteum* including Z-medium (Domnas et al. 1977), media containing hemp seed (Jaronski et al. 1983), various types of natural oils (Domnas et al. 1977) and a combination of the above (Domnas et al. 1982, Kerwin et al. 1986). The effects of storage on *L. giganteum* mycelium may vary in those media. In addition, differences in storage survival may be somewhat different for other *L. giganteum* isolates (North Carolina and Louisiana).

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