EFFECT OF NUTRIENT CONCENTRATION IN CULTURING THREE ISOLATES OF THE MOSQUITO FUNGAL PATHOGEN, *LAGENIDIUM GIGANTEUM* (OOMYCETES: LAGENIDIALES), ON SUNFLOWER SEED EXTRACT[1]

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ABSTRACT. The requirements for soluble protein concentration in sunflower seed extract (SFE) cultures were similar for 3 isolates of *Lagenidium giganteum* from North Carolina, Louisiana and California. Sunflower seed extract agar containing soluble protein in the range of 1.0 to 4.0 mg/ml was satisfactory for high levels of zoospore production. It is recommended that the protein concentration be 2.0 mg/ml for both SFE agar and liquid SFE culturing media, and the protein assay be with the Bradford method using bovine serum albumin as the standard.

INTRODUCTION

The fungus *Lagenidium giganteum* Couch is a potential biological control agent for mosquito larvae (see review by McCray 1985). Jaronski and Axtell (1984) described a procedure for mass culturing the vegetative phase of *L. giganteum* on sunflower seed extract (SFE) having a soluble protein content of 1 mg/ml. Under proper conditions, the SFE culture system is suitable for the production of high yields of infective zoospores.

Further work with *L. giganteum* culturing in SFE indicated that nutrient concentration in SFE may not be as critical for induction of zoosporogenesis as previously considered. The present experiments were conducted to determine the range of nutrient concentration in SFE that is suitable for zoospore induction and compatible with the production system. Soluble protein concentration is used as an indicator of nutrient concentration in SFE (Jaronski and Axtell 1984); therefore, 3 commonly used protein assays (Bradford, Lowry and Biuret) were evaluated to select the most simple and accurate assay for protein determination in SFE.

The SFE procedure was originally developed using a North Carolina (NC) isolate of *L. giganteum*. The NC isolate and 2 other isolates, California (CA) and Louisiana (LA), were evaluated in this study for their ability to produce zoospores and infect mosquito larvae after prolonged culturing on SFE without cycling through a host.

MATERIAL AND METHODS

The CA and LA isolates were obtained from J. Lord, Boyce Thompson Institute, Ithaca, NY and the NC isolate from A. Domnas, University of North Carolina, Chapel Hill, NC. All 3 isolates were cultured on SFE liquid media for ca. 6 months in our laboratory.

PROTEIN CONCENTRATION DETERMINATIONS. Soluble protein concentration in SFE prepared by the procedure described by Jaronski and Axtell (1984) was determined by the Lowry (Lowry et al. 1951), Bradford (Bradford 1976; Bio-Rad Chemical Division, 2200 Wright Avenue, Richmond, CA 94804) and Biuret (Henry 1974, Fisher Scientific Catalog No. SB51-500) methods. Standard curves were determined using bovine serum albumin (BSA) or lysozyme (LYS) protein solutions. Protein concentration was determined on freshly prepared, unfiltered SFE. Since the initial protein concentration was too high for the sensitivity of the protein assays, the SFE was diluted appropriately with deionized water prior to the analyses. Protein determinations were conducted at least in triplicate for each SFE sample and standard solutions. At least 5 concentrations of standard protein solution were used to construct the standard curves. Protein determinations were conducted on extracts prepared from 4 batches of sunflower seeds obtained from different food stores in Raleigh, NC.

SFE SOLID CULTURE ASSAYS. Each of the 3 isolates of *L. giganteum* was grown in liquid SFE (1 mg/ml soluble protein determined by Bradford assay using BSA as standard) for 1 week. One ml aliquots from this culture were transferred to 100 mm diam petri dishes containing ca. 10 ml of SFE agar of various protein concentrations (0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml). These plates were stored in air tight plastic bags for 4, 8 and 12 days at 15°C prior to conducting the assays. Three different assays were conducted to determine the effect of nutrient concentration on zoosporogenesis: vesicle counts, zoospore counts and infectivity rates. For each isolate, one plate from each concentration and storage period was assayed by each method and data from the 3 storage periods

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were pooled for statistical analysis to determine significant differences due to protein concentration.

VESICLE COUNTS. One 200 mm² culture disk was removed from the center of each culture plate and immersed in 20 ml of deionized water. After 6 hr, each disk was removed from the water, and observed at 100x magnification with a compound microscope. The number of mature vesicles (vesicles in which zoospores were clearly differentiated) per field of view (3.14 mm²) was recorded. Five counts were conducted on each disk and then the disks were replaced in the water. Vesicle counts were again conducted at 8, 13, 18, 28, 38, 48 and 58 hr postimmersion on each disk. As vesicle formation intervals and peaks varied among the protein concentrations, the number of vesicles was plotted with respect to time interval for each disk and the area under the resulting curve was calculated. The areas were used as a relative measurement of total number of vesicles formed from each disk.

ZOOSPORE COUNTS. Agar disks (200 mm²) were immersed in sterile deionized water as described above. One-ml samples were removed at similar intervals as for the vesicle-count assays, and immediately spread on peptone-yeast-glucose (PYG) agar plates (100 mm diam). The zoospores present in these samples encysted, germinated, and produced hyphae after 5–10 hr in the PYG plates. Each plate was then observed using a compound microscope and the number of germinated zoospores were counted in 20 fields (3.14 mm²) to determine the mean number of zoospores per field. The procedure was conducted 6, 8, 18, 28, 38, 48 and 58 hr after immersion of the disk in the water. As with the vesicle counts, the total number of zoospores per field was determined from the area under the curve of number of zoospores versus time. From these data, the number of zoospores per plate was calculated.

INFECTIVITY RATES. Bioassays with mosquito larvae were used to verify the infectivity of *L. giganteum* zoospores. The bioassay unit consisted of 100 ml of deionized water and 25 second- or third-instar larvae (4–5 mm/long) of *Culex quinquefasciatus* Say in white plastic cups (9 cm diam × 5 cm height). One 20 mm² disk of CA isolate or three 200 mm² disks of NC and LA isolates were placed in the water with the larvae. The different amounts were used because in preliminary experiments the CA isolate exhibited ca. 10-fold rate of infection compared to the NC and LA isolates. Larvae were fed daily with 0.25 ml of liver powder suspension (35 mg/ml). Daily infectivity was recorded by removing dead larvae from the cups and inspecting them for fungal infection. All assays were conducted at room temperature (20–27°C).

SFE LIQUID CULTURE ASSAYS. The three isolates of *L. giganteum* were cultured in liquid SFE with 0.2, 2.0 and 5.0 mg/ml soluble protein, for 1 week, and then transferred to agar plates (2.0 mg/ml protein) as described previously. These plates were stored at 15°C for 4 days and then assayed for their ability to produce vesicles (vesicle counts), and infect *Cx. quinquefasciatus* larvae. Four plates of each isolate were assayed for each protein concentration. Vesicle counts and bioassays with larvae were performed as described previously for the solid culture assays.

STATISTICAL ANALYSES. Analysis of variance was performed on the data to determine overall treatment differences. The data for the solid agar assays were analyzed as a completely randomized block design (block-storage period) to remove variability due to storage from the analysis since it was not of interest. The liquid culture data was analyzed as a completely randomized design with four replicates (plates). If significant differences were present, individual treatment mean comparisons were conducted using the protected Least Significant Difference test (Steele and Torrie 1980, p. 173).

RESULTS

PROTEIN CONCENTRATION DETERMINATIONS. There were no significant differences in protein concentration in SFE made from 4 different batches of seed. There were, however, significant differences in the protein concentrations determined by different assay methods and standards (Table 1). The Bradford assay was the most simple and least time-consuming procedure. The increase in turbidity after adding reagents was slight and did not interfere with the assay. Protein concentration in SFE using this method was 10.1 mg/ml using BSA as standard solution and 2.5 mg/ml when lysozyme was the standard. The Lowry assay resulted in turbidity interference when the reagents were added but a correction factor could be used since the turbidity effects were additive. With this assay, protein concentration in SFE was 14.9 mg/ml using BSA as a standard and 9.1 mg/ml when lysozyme was the standard. Data obtained with the Biuret method are not presented because there was extensive turbidity which made the results unreliable and the method not suitable for measuring protein concentration in SFE.

SFE SOLID CULTURE ASSAYS. Table 2 shows a summary of the results of the 3 assay methods with *L. giganteum* grown on SFE agar with different concentrations of soluble protein. There

Table 1. Soluble protein concentration in unfiltered sunflower seed extract (SFE) analyzed by the Bradford and Lowry procedures using bovine serum albumin (BSA) or lysozyme (LYS) as standard solutions.

Procedure	Standard	Protein (mg/ml) per seed batch[a] 1	2	3	4	Mean
Bradford	BSA	10.6b	10.7b	9.5b	9.6b	10.1b
	LYS	2.4a	3.6a	1.7a	2.1a	2.5a
Lowry	BSA	15.1c	14.8c	14.9c	14.7c	14.9c
	LYS	9.76b	9.9b	8.4b	8.2b	9.1b

[a] Each value is mean of 3 samples of SFE, 3 assays per sample. Within each column, values followed by the same letter were not significantly different (Protected LSD, P = 0.05). For each assay method and standard, the protein concentrations among the 4 batches of seed were not significantly different (Protected LSD, P = 0.05).

were significant differences (ANOVA, P = 0.01) in vesicle formation by the 3 isolates in plates of different protein concentration. Lowest vesicle formation by the CA and NC isolates occurred from plates with 0.2 mg/ml while for the LA isolate it was lowest in plates with 8.0 mg/ml protein. Highest vesicle formation by the CA isolate occurred on plates with 4.0 mg/ml protein, by the LA isolate with 2.0 and 4.0 mg/ml, and by the NC isolate with 1.0 and 2.0 mg/ml.

There were significant differences in the relative number of zoospores produced by the 3 isolates reared in SFE agar of different protein concentration. Zoosporogenesis data correlated closely with vesicle counts (r = 0.975, 0.987 and 0.935 for the CA, LA and NC isolates, respectively). The highest number of zoospores produced by the CA isolate occurred on plates with 4.0 mg/ml protein, by the LA isolate with 2.0 and 4.0 mg/ml and by the NC isolate with 2.0 mg/ml. There were significant differences (ANOVA, P = 0.01) in the percent mortality of *Cx. quinquefasciatus* larvae after exposure to the 3 isolates which had been cultured in SFE agar of varying protein concentration. Highest percent infections by the CA isolate occurred with the isolate cultured on SFE containing 1.0, 2.0 and 4.0 mg/ml protein. With the LA isolate highest infections were with 0.5, 1.0, 2.0, and 4.0 mg/ml; highest for the NC isolate was with 1.0 mg/ml.

SFE LIQUID CULTURE ASSAYS. There were significant differences (ANOVA, P = 0.01) in the number of vesicles produced after culturing in liquid SFE containing different protein concentrations. In all 3 isolates, no significant differences (P = 0.05) were detected on percent mortality of *Cx. quinquefasciatus* larvae (Table 3). Highest vesicle formation by the CA and NC isolates occurred when the liquid phase SFE contained 2.0 mg/ml protein. No difference was observed in vesicle numbers of the LA isolate after culturing in liquid media with 0.2 and 2.0 mg/ml protein but a significant reduction occurred after culturing in media with 5.0 mg/ml.

DISCUSSION

Nutrient concentration, expressed as soluble protein concentration, in SFE agar culturing medium had an overall significant effect on the

Table 2. Effect of protein concentration of SFE agar medium on the ability of 3 isolates of *Lagenidium giganteum* to produce vesicles, zoospores and infect *Culex quinquefasciatus* larvae.

Protein Concn. (mg/ml)	Mean no. vesicles per agar plate ($\times 10^4$)[a,b] CA	LA	NC	Mean no. zoospores per agar plate ($\times 10^6$)[a,b] CA	LA	NC	Percent mortality of larva[a,c] CA	LA	NC
0.2	5.80a	6.93ab	21.13a	1.73a	0.33a	0.46a	62.9a	24.9a	22.1a
0.5	12.75a	19.49bc	34.17a	3.84a	0.70ab	1.46b	81.2ab	57.7b	36.6ba
1.0	54.20b	31.26cd	92.64c	8.39ab	1.00ab	2.87bc	94.7b	62.7b	52.2c
2.0	62.98b	38.91d	93.84c	10.38ab	1.27b	3.32c	93.7b	67.4b	38.8ba
4.0	92.32c	42.89d	77.91b	15.52d	1.65b	2.42bc	94.7b	53.9b	38.9ba
8.0	56.57b	0.18a	34.30ab	11.99b	0.04a	1.87b	87.5b	28.6a	20.4a

[a] Means followed by the same letter within a column were not significantly different (Protected LSD, P = 0.05).

[b] Three plates per concentrate per isolate.

[c] Larvae exposed to 20 mm^2 agar disk from culture of CA or 600 mm^2 of LA and NC, 3 cups per concentration per isolate.

Table 3. Effect of protein concentration in SFE liquid medium on the ability of *Lagenidium giganteum* to produce zoospores and infect *Culex quinquefasciatus* larvae after subsequent culture on SFE agar plates.

Protein Concn. (mg/ml)	Mean no. vesicles per agar plate (× 10^4)[a]			Percent mortality of larvae[a,b]		
	CA	LA	NC	CA	LA	NC
0.2	39.04a	29.62a	47.94a	94.1a	78.0a	55.0a
2.0	58.16c	29.56a	72.63b	96.1a	85.0a	62.0a
5.0	49.77b	17.02b	47.32a	96.0a	75.0a	62.0a

[a] Within a column, means followed by the same letter were not significantly different (protected LSD, P = 0.05).

[b] Larvae exposed to 20 mm^2 agar disk from CA culture or 600 mm^2 of LA and NC, four cups per concentration for each isolate.

ability of the 3 isolates of *L. giganteum* to produce vesicles and zoospores. Although there were differences among the 3 isolates, the higher levels of vesicle formation and zoospore production occurred for all the isolates cultured in SFE agar with the protein concentration 1.0 to 4.0 mg/ml. For the CA isolate, vesicle formation and zoospore production were also high with the protein concentration 8.0 mg/ml although the vesicle formation was less than at 4.0 mg/ml. Therefore, the protein concentration was not as critical as previously thought. Microscopic examination revealed reduced mycelial growth and sporangial formation in the less concentrated SFE agar plates (0.2 and 0.5 mg/ml protein). At higher concentrations, mycelial growth was vigorous and fungal sporangia were densely packed on the agar medium. These observations suggest that zoosporogenesis levels were in part a function of the amount of vegetative growth present on the plates. The production of zoospores at all protein concentrations indicate that the appropriate sterols (Domnas et al. 1977) required for zoosporogenesis were present. With the bioassay method used, the optimal protein level for larval infection was 1.0 mg/ml for the NC isolate but for the CA and LA isolates, there were only slight differences in the infectivity in the range of 0.5 to 8.0 mg/ml and 0.5 to 4.0 mg/ml, respectively.

The lower levels of zoosporogenesis and infectivity at the highest protein concentration (most apparent with the LA isolate) in the SFE agar plates suggest an inhibitory mechanism was present, since there was an abundance of mycelia and sporangia on the agar. Jaronski et al. (1983) indicated that repressor compounds may exist in the medium which at high concentrations may cause inhibition of zoosporogenesis. The nature of these compounds is unknown although recent studies (Lord and Roberts 1985) indicate that certain organic compounds (sugars, amino acids and peptides) can inhibit zoosporogenesis. The 3 isolates of *L. giganteum* in this experiment responded similarly to nutrient concentration in SFE. The LA isolate was particularly sensitive to the 8.0 mg/ml protein level. At this concentration, low vesicle formation rates and zoospore densities were observed. Infection of *Cx. quinquefasciatus* larvae occurred, nevertheless, and the further dilution rate in the larval bioassay (100 ml) relative to the vesicle-count assay and zoosporogenesis assay (20 ml) could have accounted for these results. Jaronski et al. (1983) observed increased zoosporogenesis rates with increasing water volume in similar assays.

Domnas et al. (1982) have shown that increasing concentrations of nutrients in liquid media improved mycelial yields, but repressed zoospore output in the NC isolate of *L. giganteim.* Similar observations have been more recently reported by Lord and Roberts (1985) with other isolates including the CA and LA isolates. In our experiments using SFE agar, there was a proportional increase in zoospore output with increasing vegetative development up to a certain level (ca. 8.0 mg/ml) when the opposite trend became evident. In our experiment using liquid SFE with 3 protein concentrations, vesicle formation was lower with the protein concentration 5.0 mg/ml than with 2.0 mg/ml and zoospore yield was probably affected in the same way. However, the differences in vesical formation (and presumably zoospore production) with different protein concentrations did not result in any significant differences in larval mortalities in the bioassay.

Sunflower seeds contain 15 to 20% protein (Dorrell 1981). These values correspond to approximately 1.5 to 2.0 gms of protein per 200 ml of SFE broth produced following the procedure of Jaronski and Axtell (1984). This is equivalent to 7.5 to 10.0 mg/ml protein concentration. This value corresponds to the measurements of protein obtained using the Bradford assay (with the BSA standard) and the Lowry assay (with the lysozyme standard). The Bradford assay underestimated protein concentration when used with the lysozyme standard while the Lowry assay overestimated protein concentration when used with the BSA standard.

Based on these data, culturing of *L. giganteum* on SFE agar following the procedure by Jaronski and Axtell (1984) may be in SFE containing soluble protein in the range of 1.0 to 4.0 mg/ml. The optimal protein concentration for different isolates may vary within that range. We recommend that the protein level for both

the solid phase (SFE agar) and the liquid SFE phase be 2.0 mg/ml. The protein concentration should be measured using the Bradford assay and BSA as the protein standard.

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