

In Vitro Production of Zoospores by the Mosquito Pathogen *Lagenidium giganteum* (Oomycetes: Lagenidiales) on Solid Media¹

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Reliable, large-scale production of *Lagenidium giganteum* zoospores was obtained on solid media. The fungus was grown for 7 days in a liquid medium of wheat germ, hemp seed, yeast extract, and glucose, then placed onto hemp-seed agar. Zoosporogenesis was induced on agar by immersing the fungal cultures into water. Zoospore production began 10 hr postimmersion, peaked at 18 hr, and ceased by 36 hr. A single, 10-cm Petri dish of fungus on hemp-seed agar produced $1.7-3.8 \times 10^7$ zoospores during the 26 hr of zoosporogenesis. Optimal zoospore production occurred with 4- to 7-day-old cultures; cultures older than 10 days produced few zoospores. The temperature range for zoosporogenesis was 15-35°C. The extent of zoosporogenesis was directly related to the volume of water used to induce zoospore formation and inversely proportional to agar thickness. Bioassay of zoospores against second instar *Culex quinquefasciatus* larvae yielded an LD₅₀ of 400 zoospores/ml.

KEY WORDS: *Lagenidium giganteum*; zoospore; biological control; mosquitoes.

INTRODUCTION

The use of *Lagenidium giganteum* for biological control of mosquitoes requires a simple and rapid means of mass-producing infective zoospores. All earlier field evaluations of *L. giganteum* have used this fungus reared in living mosquito larvae (e.g., Umphlett and Huang, 1972; Washino et al., 1976). Such methods are time consuming, labor intensive, and pose problems in the quantification of doses.

Domnas et al. (1982) described techniques for obtaining zoospores in liquid culture. Several liquid media allowed zoosporogenesis by *L. giganteum*, with their "Z Medium" being the best. However, their techniques required a strict culturing schedule, which reduced the flexibility of

the method to produce zoospores on demand. Our purposes in the currently reported work were to determine whether any of these liquid media would enhance zoosporogenesis from hemp-seed agar, a solid medium developed by Domnas et al. (1977), to investigate various parameters affecting zoosporogenesis and to develop procedures for obtaining large number of zoospores on solid media.

MATERIALS AND METHODS

The North Carolina isolate of *L. giganteum* was routinely maintained by weekly subculture in peptone-yeast extract-glucose broth (PYG) as previously described by Domnas et al. (1974, 1977). Several liquid culture media were used for vegetative growth of the fungus prior to inoculation onto agar. These media, developed by Domnas et al. (1982), were WGYG (3.2 g wheat germ/liter, 1.4 g yeast extract/liter,

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1.2 g glucose/liter), HSYG (250 mg hemp-seed extract as soluble protein/liter, 1.4 g yeast extract/liter, 1.2 g glucose/liter) and Z Medium (an equal mixture by volume of WGYG and HSYG).

Hemp-seed agar (HSA) was prepared according to Domnas et al. (1977) with the concentration of the hemp-seed extract diluted to 1.0 mg soluble protein/ml agar medium. Thickness of HSA plates was standardized at 2–3 mm, except where noted.

Lagenidium cultures on HSA were immersed in distilled water (1 liter per agar plate) and periodically examined for sporogenic vesicles during the subsequent 48 hr. Except for temperature experiments, zoosporogenesis was studied at 25–27°C. Zoospore production was estimated by counting the number of vesicles in six 3-mm² fields on each of three agar dishes and calculating the mean number of vesicles/mm². This method was based on observations that all vesicles produced motile zoospores. Comparisons of vesicle production were made at approximately 18 hr postimmersion, which was the peak period for zoospore production.

We also investigated the effects of water volume and agar thickness on the extent of zoosporogenesis. To study the former, we immersed *L. giganteum* on HSA into different volumes of water and observed the subsequent vesicle production. Effects of agar thickness were examined by growing the fungus on HSA of varying thickness within the same Petri dish, then immersing 1 cm diameter plugs cut from the agar in 1 liter distilled water. Relative zoospore production was assayed in both experiments by the number of vesicles/mm² at the peak of zoosporogenesis.

Zoospore suspensions, ranging from 278 to 1500 zoospores/ml, were collected for bioassay 16–18 hr postimmersion. For the assay, 25 second instar *Culex quinquefasciatus* larvae were placed into 100 ml of the different zoospore suspensions. There were two replicates per zoospore concentration. The larvae were examined 48–72 hr later for the presence of *L. giganteum* hyphae within their bodies. Bioassay data

TABLE 1
COMPARISON OF ZOOSPORE PRODUCTION BY
Lagenidium giganteum ON HEMP-SEED AGAR
AFTER TRANSFER FROM DIFFERENT LIQUID MEDIA

Liquid medium	Mean ^a No. vesicles/mm ² (± SD) produced on agar cultures
WGYG	1.0 (0.69)
HSYG	2.5 (2.07)
Z Medium	
HSYG:WGYG (1:1)	10.5 (2.67)
PYG	<1.0

^a Based on counts of six 3-mm² fields on each of three HSA plates after the plates were immersed in 1 liter water each for 18 hr. Cultures were 8 days old.

were analyzed by the method of Litchfield and Wilcoxon (1949).

RESULTS

As shown in Table 1, optimum zoospore production occurred when the fungus was grown first in Z Medium and then on HSA. When either WGYG or HSYG was used alone in the liquid phase of culture, zoospore production from HSA was much lower than when these two liquid media were combined as Z Medium. Evidently wheat germ and hemp-seed extract complemented each other, for reasons unknown. When PYG was used for the liquid phase, zoosporogenesis was very slight from subsequent cultures on HSA.

Zoospore production began about 10 hr after the *Lagenidium* cultures on HSA were immersed in water. Vesicle (and zoospore) production then increased rapidly reaching a peak about 18 hr postimmersion. Production subsequently decreased and ceased by 36 hr postimmersion. Zoospore release from a single vesicle occurred about 30 min after formation of that vesicle. A mean of 11.5 zoospores (range of 2 to 20) was produced per vesicle. Basing our calculations on the mean number of zoospores per vesicle, the duration of the vesicle stage and number of vesicles/mm² of agar at intervals during the zoospore production cycle, we estimated that a typical fungal culture produced 1.7–3.8 × 10⁷ zoospores per 10-cm-diameter dish during the 26-hr production period.

Lagenidium grown more than 2 weeks in Z Medium without subculture before transfer to HSA exhibited significant decrease in zoosporogenesis. Repeated subculture through 10 transfers of Z Medium before plating onto HSA did not cause any decrease in zoospore production.

Lagenidium giganteum maintained on HSA lost its maximal potential for zoosporogenesis with increasing time of storage at 25°C (Table 2). After 6 days, the drop in zoosporogenesis became pronounced. Cultures older than 3 weeks produced very few zoospores. Storage of *Lagenidium* on agar at 4°C drastically reduced zoospore production after 1 week and killed the fungus after 2 weeks.

Zoospore production increased with the volume of distilled water used to induce zoosporogenesis (Table 3). When agar cultures of the fungus, immersed in 200 ml water, were transferred twice to 200 ml fresh water during the induction period, the zoospore production attained levels that were obtained with 1 liter. Furthermore, the thickness of the agar substrate was inversely proportional to the zoospore production. At peak production, *Lagenidium* produced an average of 27.1 vesicles/mm² from 0.75- to 1-mm-thick agar, 12.3 vesicles/mm² from 2- to 3-mm-thick agar, and 5.5 vesicles/mm² from 6- to 7-mm-thick agar. Times of onset and peak of zoospo-

rogenesis were not appreciably different among the *Lagenidium* cultures grown on the three thicknesses of agar.

Water from the local municipal supply did not induce zoosporogenesis, for reasons unknown. Water from a local farm pond induced zoospore production to approximately the same extent as distilled water.

Zoosporogenesis had upper and lower limits of 35° and 15°C, respectively, with an optimum temperature range of 21–27°C (Table 4).

An LD₅₀ of 400 zoospores/ml with 95% confidence limits of 270–591 zoospores/ml was obtained from the assay. The slope function of the log-dose probit regression was 3.82. Heterogeneity of the plotted data was not significant (χ^2 analysis, $P = 0.05$), thus the plotted regression was a good fit.

DISCUSSION

Optimum zoospore production was obtained when *L. giganteum* was first grown for 1 week in Z Medium, then 4–7 days on HSA, before induction of zoosporogenesis. Induction was achieved by immersing the fungal cultures in water.

A *Lagenidium* culture on a 10-cm-diameter Petri dish of HSA produced 1.7–3.8 × 10⁷ zoospores. Umphlett and Huang (1972) estimated that an infected mosquito larvae produced 1.8 × 10⁵ zoospores, based on their counts of sporangia per larva and zoospores per vesicle. (We believe that their values are overestimates because we observed that many cadavers were only partially filled with sporangia and that not all sporangia produced vesicles.) Thus, one agar plate of fungus produced by our method yielded at least 100 larval equivalents of zoospores. Seventy-five milliliters of Z Medium yielded mycelium for 50 Petri dishes, or 8.5–19 × 10⁸ zoospores. We were unable to compare the infectivity of zoospores produced in vivo with that of zoospores from HSA because not enough zoospores could be collected at any one time from infected mosquito larvae for an assay. We did observe numerous infections initiated by one or two zoospores in our

TABLE 2

EFFECT OF STORAGE TIME OF AGAR CULTURES AT 25–27°C ON ZOOSPORE PRODUCTION BY *Lagenidium giganteum*

Storage period (days)	Mean ^a No. vesicles/mm ² (± SD)
4	35.1 (3.8)
5	22.5 (3.7)*
6	14.4 (3.5)*
11	10.2 (2.1)*
12	6.3 (1.0)*
17	2.2 (0.8)*
18	3.4 (0.6)*
24	0.5 (0.3)*
26	0.3 (0.3)*
30	0

^a See footnote a, Table 1.

* Significantly different from vesicle production by the 4-day culture ($P = 0.01$).

TABLE 3
EFFECT OF WATER VOLUME ON ZOOSPORE PRODUCTION BY *Lagenidium giganteum*

Volume H ₂ O (ml)	Mean ^a No. vesicles/mm ² (± SD) after immersion for			
	12 hr	15 hr	18 hr	21 hr
200	0	1.8 (0.5)*	12.8 (1.4)*	9.0 (2.3)*
500	1.8 (0.5)	18.7 (2.3)	17.8 (1.7)*	15.2 (1.5)*
1000	2.0 (0.5)	18.1 (1.8)	39.4 (5.5)	26.1 (1.7)
200 ^b	3.0 (0.5)	16.2 (1.4)	37.5 (4.2)	29.7 (1.9)

^a Based on counts of six 3-mm² fields on each of three HSA plates for each volume of water and time period.

^b Water replaced twice at 3-hr intervals.

* Significantly different from the 1000-ml treatment for the corresponding time interval ($P \leq 0.01$, Mann-Whitney rank sum test).

assays and therefore believe that zoospores produced in vitro are just as infective as zoospores from larvae. Zoospores produced on HSA have been successfully used in limited field release of *L. giganteum* against *Cx. quinquefasciatus* (Jaronski and Axtell, 1982).

Prolonged storage seriously affected zoospore production and limited the shelf life of cultures on HSA. *Lagenidium giganteum* cultures older than 10 days were useless for large-scale production of zoospores. Despite this limitation, the use of

HSA is a marked improvement over the strict schedule of zoospore production in liquid culture and provides a solid substrate for distributing *Lagenidium* in the field.

The temperature limits for zoosporogenesis are important because temperatures of larval habitats may exceed 29°C, particularly in warm temperate and tropical climates. The fungus is likewise limited in usefulness against mosquitoes breeding in water cooler than 20–21°C, e.g., in boreal regions. This temperature limitation, along with the adverse affects of organic pollution (Jaronski and Axtell, 1982) and salinity (Merriam and Axtell, 1982), define several important parameters within which *L. giganteum* has potential usefulness as a mosquito control agent.

In our protocol, the Z Medium provides the necessary energy sources, vitamins, and sterols during vegetative growth of the fungus. The sterols are particularly essential for subsequent zoosporogenesis and must be supplied during vegetative growth because the addition of sterols to the water during induction of zoosporogenesis has no effect (Domnas, unpubl.). Hemp-seed extract seems to be the best source of sterols for reasons that still remain unclear. Hemp-seed agar continues to provide the sterols and limited nutrients while serving as a solid substrate for zoosporogenesis. The agar is not essential because *L. giganteum* will produce zoospores when transferred directly from Z Medium to water (Domnas et al., 1982).

TABLE 4
EFFECT OF TEMPERATURE ON *Lagenidium giganteum* ZOOSPOROGENESIS AS MEASURED BY TIME OF ONSET AND PEAK AFTER IMMERSION OF HEMP SEED AGAR PLATE IN WATER VOLUME AND THE VESICLE DENSITY AT PEAK

Temperature (°C)	Time of vesicle formation		Mean ^a No. vesicles/mm ² (± SD) at peak
	Onset (hr)	Peak (hr)	
10	—	—	0
15	36	48	2.2 (1.8)*
19	36	40	4.5 (1.2)*
21	18	24	25.5 (6.4)
27	10	18	30.2 (2.0)
32	10	18	1.7 (1.2)*
35	10	12	0.2 (0.3)*

^a Based on counts of six 3-mm² fields on each of three HSA plates after the plots were immersed in 1 liter water each.

* Difference from 27°C treatment significant at the 1% level (Mann-Whitney rank sum test).

Immersion of the fungal cultures in water evidently causes leaching of zoosporogenic repressor(s) from the cultures. This leaching process is related to the amount of agar and the volume of elutant. Zoospores are produced abundantly in vitro only when all the requirements of the fungus are met: ample nutrients plus sterols during vegetative growth, followed by the elution of repressor(s). These events probably mimic events within an infected larva and are paralleled by conditions necessary for sporogenesis in other Oomycetes such as *Aphanomyces astaci* (Unestam, 1969), *Lagenidium callinectes* (Bland and Amerson, 1973), and *Phytophthora cinnamoni* (Byrt and Grant, 1979).

The identity of the repressor(s) is as yet unknown. These substances may be amino acids (Domnas, unpubl.). We are currently attempting to identify these compounds and define their mode of action.

REFERENCES

- BLAND, C. E., AND AMERSON, H. V. 1973. Observations on *Lagenidium callinectes*: Isolation and sporangial development. *Mycologia*, **65**, 310–320.
- BYRT, P., AND GRANT, B. R. 1979. Some conditions governing zoospore production in axenic cultures of *Phytophthora cinnamoni* Rands. *Aust. J. Bot.*, **27**, 103–115.
- DOMNAS, A. J., FAGAN, S. M., AND JARONSKI, S. T. 1982. Factors influencing zoospore production in liquid culture of *Lagenidium giganteum* (Oomycetes: Lagenidiales). *Mycologia*, **74**, 820–825.
- DOMNAS, A. J., GIEBEL, P. E., AND MCINNIS, T. M. 1974. Biochemistry of mosquito infection: Preliminary studies of biochemical change in *Culex pipiens quinquefasciatus* following infection with *Lagenidium giganteum*. *J. Invertbr. Pathol.*, **24**, 293–304.
- DOMNAS, A. J., SREBRO, J. P., AND HICKS, B. F. 1977. Sterol requirement for zoospore formation in the mosquito-parasitizing fungus *Lagenidium giganteum*. *Mycologia*, **69**, 875–886.
- JARONSKI, S. T., AND AXTELL, R. C. 1982. Effects of organic water pollution on the infectivity of the fungus *Lagenidium giganteum* (Oomycetes; Lagenidiales) for mosquito larvae (*Culex quinquefasciatus*): Field and laboratory evaluation. *J. Med. Entomol.*, **19**, 255–262.
- LITCHFIELD, J. T., AND WILCOXON, F. 1949. A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.*, **96**, 99–113.
- MERRIAM, T. L., AND AXTELL, R. C. 1982. Salinity tolerance of two isolates of *Lagenidium giganteum* (Oomycetes; Lagenidiales), a fungal pathogen of mosquito larvae. *J. Med. Entomol.*, **19**, 388–393.
- UMPHLETT, C. J., AND HUANG, C. S. 1972. Experimental infection of mosquito larvae by a species of the aquatic fungus *Lagenidium*. *J. Invertebr. Pathol.*, **20**, 326–331.
- UNESTAM, T. 1969. On the physiology of zoospore production in *Aphanomyces astaci*. *Physiol. Plant.*, **22**, 236–245.
- WASHINO, R. K., FETTER, J., FUKUSHIMA, C. K., AND GONOT, K. 1976. The establishment of *Lagenidium giganteum*, an aquatic fungal parasite of mosquitoes, three years after field introduction. *Proc. Papers Calif. Mosq. Control Assoc.*, **44**, 52.