SIMPLIFIED PRODUCTION SYSTEM FOR THE FUNGUS *LAGENIDIUM GIGANTEUM* FOR OPERATIONAL MOSQUITO CONTROL[1]

S. T. JARONSKI AND R. C. AXTELL[2]

Department of Entomology, North Carolina State University, Raleigh, NC 27695–7613

ABSTRACT. A protocol is presented for the production of *Lagenidium giganteum*, a fungal pathogen of mosquito larvae, using a two-phase system consisting of aqueous extracts of sunflower seed in liquid medium and in solid agar-base medium. The cultures on solid medium are applied in the field and may be stored for up to 12 weeks at 15–16°C. Suggestions are made for field use and evaluation of the fungus for mosquito control. Directions for media preparation and quality control are included.

The oomycete fungus, *Lagenidium giganteum*, has shown excellent potential as a biocontrol agent of mosquitoes (Axtell et al. 1982, Fetter-Lasko and Washino 1983). In suitable habitats, *Lagenidium* is able to infect and kill a substantial proportion of larval mosquito populations while recycling after initial introduction and surviving periods of drought and low temperatures (Jaronski and Axtell 1983a). Relatively complex laboratory culturing methods suitable for limited production for research purposes are available (Domnas et al. 1982, Jaronski et al. 1983). However, if this fungus is to be used by mosquito abatement districts and other organized mosquito control programs, simplified protocols for mass production, harvest and application in the field are needed. Such a simplified protocol, selected after evaluations of a variety of culturing media and methods, is described.

The protocol for mass production of *Lagenidium* consists of a two-phase system (Fig. 1): (1) Large amounts of vegetative mycelium are produced in a liquid medium and (2) the mycelium is transferred to solid agar-base medium for storage until used in the field. This biphasic system is superior to one consisting solely of liquid media because the fungus can be stored for several weeks on agar media, which allows flexibility in synchronizing production of fungus and its introduction into habitats with mosquito breeding.

PREPARATION OF MEDIA

An aqueous extract of edible sunflower seed (*Helianthus annus*) is recommended as a readily available, inexpensive medium for production of this fungus, although it may be possible to substitute other substances such as certain types of beans (Jaronski et al.)[3]. Shelled, unroasted sunflower seeds can be obtained from local health food stores or commercial distributors. The fungus readily produces zoospores (the stage which infects mosquito larvae) when grown on sunflower seed extract which apparently provides the necessary sterols.

PREPARATION OF SUNFLOWER SEED EXTRACT (SFE). The shelled seeds are pulverized to a fine flour in a blender or ball-mill. This flour is mixed with deionized or distilled water at the rate of 10 g/100 ml water and mixed in a blender for 60 sec. A common electric kitchen blender (e.g. Waring®, Osterizer®) is satisfactory. The resulting fine suspension is filtered through 6 layers of coarse cheesecloth. The residue in the cheesecloth should be squeezed dry, resuspended in another 100 ml water and filtered through the same cheesecloth. The two filtrates are then combined. This extract (SFE) consists of microscopic particles of seed and should have the appearance of light brown cream. The extract may be frozen until used, preferably in small aliquots to avoid repeated thawing and freezing when portions are needed.

If the above procedure is followed, 10 g of sunflower seed flour in the 200 ml of water should routinely yield approximately 11.5 mg soluble protein/ml extract. However, the protein content should be assayed by the Lowry Test (Lowry et al. 1951) or other colorimetric protein test such as the convenient "Bio-Rad Protein Assay" (Bio-Rad Laboratories, Box 708, Rockville Center, NY 11571). Such assays are routine in hospital laboratories or biomedical diagnostic services, which may be willing to perform the assays for reasonable cost. If protein analysis is not possible, the value of 11.5 mg/ml

[1] Paper No. 9228 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27650. This research was supported in part by NIH Grant 1R01 AI17024 and 1R01 AI 20886.

[2] Send reprint requests to second author (RCA). Present address of first author (STJ): Dept. 910, Abbott Laboratories, North Chicago, IL 60064.

[3] Jaronski, S. T., S. M. Fagan and A. J. Domnas. Simple media for zoospore production by the mosquito pathogen *Lagenidium giganteum* (Oomycetes: Lagenidiales). Submitted to Entomophaga.

may be assumed as the basis for subsequent dilutions required in media preparation.

CULTURE MEDIA. The liquid culture medium consists of SFE diluted with sufficient deionized or distilled water to contain 1 mg protein/ml. This is a 1:11 dilution of the stock SFE:water if the SFE has a protein content of 11.5 mg/ml. If assay reveals a different protein content then the dilution should be adjusted accordingly. The medium is added to cotton-stoppered 500 ml Ehrlenmeyer flasks (or similar vessels) containing 200 ml medium or 1 liter flasks with 500 ml medium and sterilized in an autoclave for 15 min at 15 psi (a pressure cooker may be used for this purpose).

The solid phase medium consists of the diluted SFE, with 20 g agar added per liter. After autoclaving, the medium should be cooled to approximately 50°C (just tolerably warm to the touch) before it is poured as a 3–4 mm thick layer of agar in sterile petri dishes or other suitable containers. Care should be taken to maintain the sterility during pouring of agar by working in sterile conditions, flame sterilizing the mouth of the flasks, and only partially lifting the covers of the petri dishes.

For the routine maintenance of reserve cultures of the fungus and for quality control, the use of peptone-yeast-glucose (PYG) broth is recommended. This medium consists of 1.2 g/liter peptone, 1.2 g/liter yeast extract, and 3.0 g/liter glucose in deionized or distilled water. Contaminants in the PYG cultures will become readily apparent in this medium. Bacteria will turn the clear medium turbid within a few days. Commonly present fungal contaminants will quickly appear as dark spherical colonies in the fluid and as dark or pigmented growth on the neck of the flask. Uncontaminated cultures will be clear with white to light tan mycelia of *Lagenidium* in irregular shapes and loose hyphal cells. Contaminants are not readily seen in SFE liquid culture because the medium is so turbid.

CULTURING PROCEDURES

STOCK CULTURES. Storage of *Lagenidium* stock cultures is on PYG agar, either as slants in tubes or in petri dishes. PYG agar consists of PYG plus 20 g agar/liter of medium. Transfer of pieces of fungal mycelium (under sterile conditions) to fresh agar medium every 4–6 weeks is sufficient for long term maintenance of *Lagenidium* stocks. Cultures are stored at 20–25°C either in the dark or light. A source of initial stock cultures is the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD, 20852, USA (Accession no. 48337 for the North Carolina strain, 48336 for the Louisiana strain and 52675 for the California strain.)

LIQUID CULTURE. The following schedule is recommended (Fig. 1):

1. Initial propagation of *Lagenidium* from stock cultures is carried out in liquid PYG (start-up culture in Fig. 1). Several small pieces of agar with mycelium should be transferred (under sterile conditions) from the stock to 100 ml culture (autoclaved) PYG in a 250 ml flask and incubated at 25–30°C with agitation (see below) for one week. The liquid PYG allows rapid, profuse growth of *Lagenidium* from agar pieces, however, zoospores are not produced and the fungus is not suitable for direct inoculation in the field. Due to the rapid growth of the fungus, it is recommended that a culture of *Lagenidium* be kept in liquid PYG as a reserve culture (Fig. 1) during the production period and used to restart SFE cultures when necessary (e.g., after contamination of SFE cultures is suspected).

2. After 1 week, 3–5 ml of the liquid PYG culture is transferred to 500 ml autoclaved, liquid SFE medium (production culture in Fig. 1). At the same time 1–2 ml of culture is also transferred to 100 ml fresh, autoclaved PYG, to create a reserve culture. The aliquots of the liquid PYG culture should be taken after agitating the culture to assure distribution of the fungal mycelium throughout the medium.

3. Subsequent subcultures of SFE and PYG cultures are made weekly. The PYG reserve cultures serve as a source of inoculum for rapid starting of SFE cultures should the latter become contaminated. All cultures are incubated at 25–30°C.

4. One-week-old *Lagenidium* cultures in liquid SFE medium are used to inoculate the solid medium as described below.

The liquid cultures (PYG and SFE) must be gently agitated continuously. Agitation of culture flasks on a rotary shaker table is standard procedure in mycological facilities. Magnetic stir-plates with inclusion of a magnetic stir bar in the culture flask are suitable substitutes, however. A standard, 38–51 mm long stirring bar in a 250–1000 ml flask will create sufficient agitation on a mechanically or hydraulically driven stir-plate. Commercial motor-driven magnetic stir-plates may not be suitable because of the heat many generate during prolonged operation. A system in which the motor is connected to the rotating magnet by pulley or a water-driven system is preferable. Water-driven stir plates can be coupled to a commercial, submersible, centrifigal pump, such as used to power water fountains, to create an economical, efficient, self-contained stirring apparatus.

Lagenidium cultures in liquid SFE or PYG

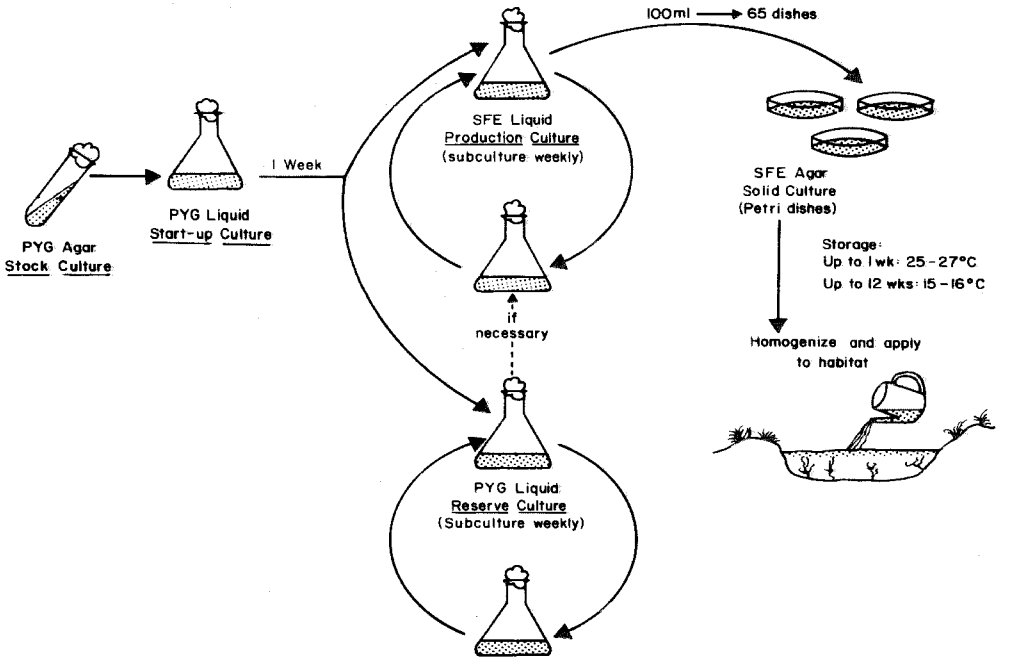

Fig. 1. Diagram of simplified production system for *Lagenidium giganteum*.

should be subcultured weekly, but the interval between transfers may be extended to 10 days if necessary. The inoculum for subculture should consist of small colonies or a fine suspension of hyphal cells. If large balls of the fungus are present in the culture, they should be broken by briefly homogenizing the contents of the flask in a sterile blender and an aliquot of the homogenate transferred to fresh medium.

Low level bacterial contamination may be controlled by subculturing the fungus into PYG containing 0.5 g/liter penicillin G and 30 mg streptomycin (added as dry powder to autoclaved PYG), incubating for 1 week, then subculturing back into antibiotic-free PYG. Prolonged use of antibiotics as insurance against contamination can mask poor asceptic technique and is not advisable.

SOLID CULTURE. One hundred ml of liquid SFE culture are sufficient to inoculate 65 petri dishes (10 cm diam.). The amount of liquid culture should be adjusted according to the number of petri dishes that will be needed. One-week-old *Lagenidium* cultures in SFE are allowed to settle by placing the flasks in a slightly tilted position. Most of the fluid is aseptically poured out and replaced with an equal volume of sterile, deionized water. This washed culture is homogenized in a blender for 10 sec.

Petri dishes of SFE agar are inoculated with 1.5 ml of the homogenate, which is spread over the entire agar surface by gentle tilting and swirling of the dish. After inoculation the plates are left overnight on a level surface to allow fungal cells to settle onto the agar and excess water to be absorbed. The petri dish cultures are then placed in plastic bags and stored. *Lagenidium* on SFE agar may be stored at 20–25°C for a maximum of 10 days without substantial loss of infective material. These cultures should be used 3–7 days after inoculation, however. At 15–16°C the fungus cultures retain their full potential for zoospore production for as long as 12 weeks. Cultures are kept in closed (tied) plastic bags during storage to prevent desiccation of the agar.

FIELD APPLICATION. Based on our field trials, an initial dose of 2 petri dishes of SFE agar culture of *Lagenidium* per m^2 of water surface should be adequate in areas where mosquito larvae are observed. The portion of a habitat containing larvae should be determined and the amount of that area used in calculating the dose needed. This will assist in conserving fungal

material and in placing the fungus in proximity to most of the larval population.

For application into mosquito breeding habitats, the solid SFE-agar cultures are homogenized in deionized water in a proportion of 1 dish per 50–100 ml water in a blender operated at medium speed for 20–30 sec. The resulting suspension will consist of small agar pieces containing the fungus. The suspension of fungus and agar can then be dispensed by hand sprayer without nozzle, or by a watering can. Homogenization of the cultures is necessary to achieve adequate dispersal of the fungus in the mosquito habitat for initial high infection rate. Continuous agitation of the suspension is necessary during field application. The agar pieces will rapidly settle otherwise and a very uneven distribution of the fungus will result.

Lagenidium will begin producing zoospores 12 hr after homogenization in water. Therefore, the suspension should be introduced into target sites within 12 hrs after preparation for best results. During transport the fungus suspension should be kept at 20–25°C. Temperatures above 30°C will rapidly kill the fungus.

Concurrent with, or prior to, field introduction of *Lagenidium* bioassays of the inoculum should be conducted to ensure that the cultures are infective and viable. A convenient assay consists of several containers, each with 1 liter deionized water and 25 second- or third-instar mosquito larvae. A suspension of homogenized *Lagenidium* agar culture is added to each container at the rate of 1/2, 1/4, 1/8, 1/16 petri dish of culture per container. One container of larvae should be left untreated, as a control. The larvae are examined for infection after 48–72 hr. Evidence of infection will be growth of mycelium in the head region initially (Fig. 2). Subsequently there will be proliferation in the thorax which will become filled with mycelia, sporangia and dark-colored spherical oospores. Larval mortality should occur within 72 hr after the start of the bioassay. Actual infection rate may vary, but if few larvae are infected at the 1/8th dish dose, the fungal inoculum has lost significant viability/infectivity and should not be used.

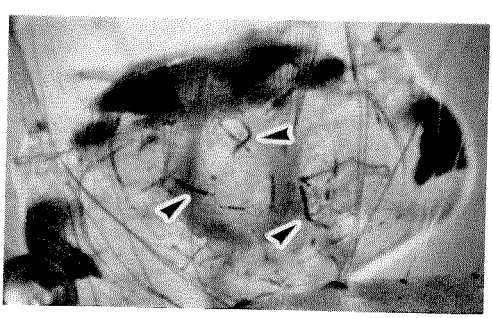

Fig. 2. Head of mosquito larvae (*Culex quinquefasciatus*) showing (arrows) the characteristic mycelium of *Lagenidium giganteum*. The dark strands of mycelium have been melanized by the mosquito.

Evaluation of the effectiveness of the fungus introduction in the field may be made by standard dipping methods to compare the numbers of live larvae to a similar untreated area several days after treatment. To determine that the larvae actually were infected, some larvae and water may be taken from the site 2–3 days after fungal introduction and observed for signs of infection and mortality. Alternative ways of evaluating the results of the fungus introduction are: 1) to take water from the sites and immediately add mosquito larvae from a laboratory colony or an untreated site and hold for observation, or 2) to place mosquito larvae in fine mesh floating containers in the site and observe the larvae for signs of infection and mortality.

Several environmental variables in the target sites need to be considered before introduction of *Lagenidium*. Salinity should not exceed 0.5 ppt. (Merriam and Axtell 1982). Organic pollution, especially from animal manure, should be very slight or absent (Jaronski and Axtell 1982). Ammonia-nitrogen greater than 10 ppm will prevent the fungus from establishing itself in the mosquito population. Water temperatures should be between 20 and 30°C for the greater part of the day (Jaronski and Axtell 1983b). Temporary breeding habitats which dry within 3 days of introduction of *Lagenidium* should not be treated with the fungus, since there will be insufficient time for the fungus to cause larval mortality and become established in the habitat.

The fungus is likely to be effective in relatively clean water habitats such as flood plain pools, ditches, and irrigation systems. Use of the fungus in a wide variety of habitats with different mosquito species will determine the practicality and suitability of using *Lagenidium* in a particular mosquito abatement program. Undoubtedly some abatement programs will find the fungus a suitable biological control agent in their area while others will not. Hopefully this protocol for *Lagenidium* production will facilitate practical evaluations of the fungus in mosquito control programs.

References Cited

Axtell, R. C., S. T. Jaronski and T. L. Merriam. 1982. Efficacy of the mosquito fungal pathogen, *Lagenidium giganteum* (Oomycetes: Lagenidiales). Proc. Calif. Mosq. and Vector Cont. Assoc. 50:41–42.

Domnas, A. J., S. M. Fagan and S. T. Jaronski. 1982. Factors influencing zoospore production in liquid cultures of *Lagenidium giganteum* (Oomycetes: Lagenidiales). Mycologia 74:820–825.

Fetter-Lasko, J. and R. K. Washino. 1983. In situ studies on seasonality and recycling pattern in California of *Lagenidium giganteum* Couch, an aquatic fungal pathogen of mosquitoes. Environ. Entomol. 12:635–640.

Jaronski, S. T. and R. C. Axtell. 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.

Jaronski, S. T. and R. C. Axtell. 1983a. Persistence of the mosquito fungal pathogen *Lagenidium giganteum* (Omycetes: Lagenidiales) after introduction into natural habitats. Mosq. News 43:332–337.

Jaronski, S. T. and R. C. Axtell. 1983b. Effects of temperature on infection, growth and zoosporogenesis of *Lagenidium giganteum*, a fungal pathogen of mosquito larvae. Mosq. News 43: 42–45.

Jaronski, S. T., R. C. Axtell, S. M. Fagan and A. J. Domnas. 1983. In vitro production of zoospores by *Lagenidium giganteum* (Oomycetes:Lagenidiales) from solid media. J. Invertebr. Pathol. 41:305–309.

Lowry, O. H., N. J. Rosebrough, A. L. Earr and R. J. Randall. 1951. Protein measurement with the Folin Reagent. J. Biol. Chem. 193:265–274.

Merriam, T. L. and R. C. Axtell. 1982. Salinity tolerance of two isolates of *Lagenidium giganteum* (Oomycetes:Lagenidiales), a fungal pathogen of mosquito larvae. J. Med. Entomol. 19:388–393.