CHAPTER 2

MATERIALS AND METHODS

Materials

A. The maintenance of fungal material

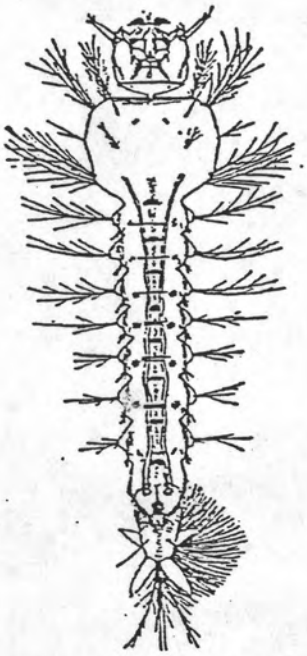
Lagenidium giganteum, the fungus, was adopted for this study. The initial culture of *L. giganteum* was obtianed from the American Type Cultures Collection (ATCC), Maryland, USA., the collection number 36942. Routinely maintained on Peptone-Yeast extract-Glucose (PYG) agar and in PYG liquid broth (see appendix). The fungus was cultured in PYG medium, 100 ml in 250 ml Erlenmeyer flasks, and maintained the fungal culture on a Gyrotary shaker at 150 rpm for few weeks, and transferred when fresh cultures were needed.

B. Preparation of mosquito materials

Species of mosquitoes used in this experiment are vectors of the local important diseases in Thailand : *Anopheles dirus*, the vector of malaria; *Aedes aegypti*, the vector of viral diseases i.e., dengue haemorrhagic fever and *Culex quinquefasciatus*, vector of filariasis and are annoyance mosquitoes. All species were maintained in the insectary at AFRIMS, Bangkok, Thailand. The procedures for continuous mosquito cultures were as follows:

EGGS

(Eggs of Anopheles)

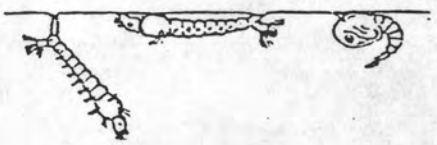


LARVA

(Larva of Anopheles)

ADULTS

(Normal position of Culex and Anopheles on the wall)



PUPAE

(Normal position of the larvae of Culex and Anopheles in the water. Culex, left; Anopheles, middle, Culex pupa, right)

Diagram 2 Life cycle of mosquitoes

1. Anopheles dirus

Each mated female, An. dirus was placed in a 9 diam. vial, 1/3 filled with water and plugged with cotton. Eggs deposited in these vials and all subsequent eggs laid in the colony were transferred with a small camel's hair brush from the oviposition container to cups of clean water. The eggs were placed inside plastic straw circles to keep them from adhering to the sides of the cup. Within a few hours after hatching, larvae were transferred to plastic pans (19.5 x 26.5 x 5 cm.) containing 800 ml of water. Approximately 200 larvae were placed in each rearing pan. The insectary temperature was 24 - 28°C and the relative humidity ranged from 55 to 90%. Windows in the insectary allowed for natural light.

The larval food was sprinkled on the water in the culture pans until the particles ceased to move rapidly across the surface. First and second stage larvae were fed twice daily. Feeding was increased to 3 - 4 times daily after most of the larvae reached third instar. Before each feeding, floating clumps of excess food were removed by dragging a paper towel across the water surface. Any larvae entrapped on the towel during the cleaning process were dislodged by rinsing the towel in a pan of clean water larvae were returned to the appropriate rearing pan.

Pupae were removed from pans twice daily and transferred to containers of water in emergence cages. Initially, adults were allowed to emerge in small cages (21 x 21 x 21 cm.). Adults, both sexes, were continuously provided a multi - vitamin syrup diluted to 40% with tap water. Two days after emergence, females were permitted to feed

on a hamster overnight. The day later, engorged females were mated with 3 - 4 days old males using the insemination system - artificial mating. After mating, the females were placed in a cage in which cups of deionized water had been placed for oviposition.

Mated females were fed a hamster nightly after the first oviposition. Eggs were collected and counted each morning. Anopheles females were allowed to oviposit on moist filter papers, which were removed at 24 hr. intervals.

After removal of the larvae resulting from this initial hatch, the eggs were returned to the water to which was added 5 mg. of pulverized rat chow. At 24 hr. intervals, newly emerged larvae were removed, counted and established as described above. Larvae were maintained at 27 ± 0.5°C. After 1 week, the first group of pupae were seen. Synchronized adult stage was obtained by selecting the pupal stage collected in the same cage. When they emerged the adults were grouped in one cage according to hatching time. The cages were maintained at 25 C and provided with water and multi - vitamin syrup for 4 - 5 days before a blood meal on hamster was provided. Eggs were counted to evaluate fecundity.

2. Aedes aegypti

The eggs of Ae. aegypti were collected by placing some pieces of paper towel inside the plastic collecting cup which was half filled with water. The mated females of Ae. aegypti would lay eggs upon the paper towel. The eggs were collected everyday, and replaced in the plastic tray (19.5 x 26.5 x 5 cm.) which was

filled with 800 ml of water. The Aedes eggs can be kept for 1 month by drying them at room temperature. After hatching, the larvae were fed with 2 - 3 tablets of food twice a day until they grew up to the pupal stage. The pupal were collected and kept in the plastic cup and 2/3 cup of water was added. The number was confirmed by recounted when they were used, then were put in the cage (42 x 42 x 42 $cm.^3$). Multi - vitamin syrup was added as food supplement for adult. Blood was necessary for producing eggs, so after reaching adult stage, alive hamster was put inside the cage for three hours or until fed mosquitoes were seen.

Females in the first gonotrophic cycle and less than 2 weeks old were allowed to oviposit on moist filter papers, which were removed at 24 hr intervals. Eggs collections were kept in 25 $\pm$ 2 $^{\circ}C$ and more than 50% relative humidity. In this condition, eggs were kept for up to 10 wk before being hatched. The normal eggs were counted and tablets of food were provided. After 24 hours, the first group of larvae was hatched. For synchronization of larvae, the filter papers with eggs were removed after the initial larvae hatched and returned to the new containers with water, and 2 - 3 tablets of food were added. At 24 hour intervals, newly emerged larvae were removed, counted and kept for experiments.

3. Culex quinquefasciatus

The adults of Cx. quinquefasciatus were cultured in laboratory cages. After feeding them once guinea pig after a week, they lay eggs. The eggs were collected and transferred with a small camels hair brush in plastic trays sized, 19.5 x 26.5 x 5 $cm.^3$,

containing 800 ml of water. Approximate 5 eggs rafts per tray were transferred. When larvae emerged and hatched later on in 7 days, tablets of food were to the larvae. The pupae were collected everyday into plastic cups and kept in new cage. The adult Culex quinquefasciatus, like other mosquito addults, were continuously provided with multi-vitamin syrup. The larvae were counted as they were being used.

C. Production of invertebrate cell lines

The cell line used in this experiment was a mosquito cell line prepared from tissues of Aedes albopictus and was maintained at Virology Department, Armed Force Research Institutes of Medical Science (AFRIMS), BAngkok, Thailand. This cell line was coded with the serial number of C6/36. For the routine maintenance of stock cultures of the C6/36, the cell were subcultured weekly in RPMI 1640 mixed with 10% Foetal calf serum in 75 $cm.^2$, corning plastic culture flasks, and incubated at 28°C.

D. Equipment and chemical reagents

1. Equipment for invertebrate cell cultures

1.1 "ISSCO" Laminar flow model H -122 (Cambridge, International Scientific Supply Co., Ltd.

1.2 25 cm^3 "Corning" plastic culture flasks (Corning, Lab ware. USA.)

1.3 Foetal calf serum (Difco, Inc., USA.)

1.4 Inverted microscope (Olympus model IMT-2, Japan)

1.5 RPMI 1640 medium (Difco, Inc., USA.)

2. Equipment for histopathological techniques

2.1 TISSUE Processor (TISSUE-TEK II) Model 4640-B Serial (Sakura Finetechnical Co., Ltd., Tokyo, Japan.

2.2 Rotary Histostat model 820 (Reichart Scientific Instruments, Buffalo, N.Y. USA.

2.3 Microtome knife (Lipshaw Many Manufacturing Company. Detroit, Mich., USA.)

2.4 Tissue-Tek water bath model 4674 serial (Miles Scientific, Laboratories, Inc., lL., USA.

2.5 'Olympus' Microscope model BHS (Olympus, Inc., Japan).

3. Equipment for bioassays

3.1 Gyrotary shaker model G 10 (New Brunswick Scientific Co., Inc., Edison, N.J., USA.)

3.2 Autoclave Model HA-3D (Hirayama Mfg. Corp., Japan)

3.3 Psychroterm Controlled Environment Incubator Shaker (New Brunswick Scientific Co., Inc., USA.)

3.4 Ace Homogenizer model Am - 1 (Nihonseiki Kaisha Ltd., Japan.)

3.5 National Blender model MX - 340 N (Matsushita Electric, Japan.)

3.6 Beckman pH meter model 70 (Beckman Instruments, Inc., USA.)

3.7 Spectronic 21 (Bausch & Lomb, USA.)

3.8 Balance model P115 (Boeckel Co., West Germany.)

3.9 Wheat germ (United Flour Mills, Ltd., Thailand.)

3.10 Sunflower seed (Local food store)

3.11 Hemp seed (Local store)

4. Media

4.1 Peptone - Yeast extract - Glucose (PYG), see appendix

4.2 Wheat germ - Yeast extract - Glucose (WGYG), see appendix

4.3 Hemp seed extract (HS)

preparation : put 10 grams of Hemp seed into 100 ml of deionized water, blended, stirred in a water - bath at 80 °C for 3 hours, then filtered and autoclaved at 15 psi, 121 °C for 15 minutes.

4.4 "Z" medium

preparation : HS from (4.3)was added with 1.4 g/l yeast extract and 1.2 g/l of glucose (HSYG). 1 part of HSYG was mixed with 1 part of WGYG (4.2) Autoclaved at 15 psi for 15 minutes.

4.5 Sunflower seed extract (SFE)

preparation :

4.5.1 The shelled seeds of sunflower were pulverized to a fine flour in a blender,

4.5.2 This flour was mixed with deionized water at the rate of 10 g/100 ml water and mixed in a blender for 30 seconds,

4.5.3 The resulting fine suspension was filtered through 6 layers of gauze pads,

4.5.4 The residue in the gauze pads was squeezed dry, resuspended in another 100 ml water and filtered through the same gauze pads. The two filtrates were then combined,

4.5.5 The extract was frozen until used,

4.5.6 The protein content of the extract was assayed by the "Bio-Rad Protein Assay," (see appendix).

Methods

A. Study of L. giganteum for propagation in the invertebrate cell culture

1. Cultured the invertebrate cell lines

The methods for cell culture and maintenance were followed the medthod of Varma et al. (1974). Stock cultures number C6/36 were grown at were grown at 28°C in 75 cm^2 corning - plastic culture flasks in 12 ml of RPMI 1640 medium containing 10% added inactivated foetal calf serum (FCS) without antibiotics. Subcultures were made weekly by seeding new flasks with cell suspensions prepared by scraping the monolayer with a rubber policeman and vigorous pipetting. Monolayers for isolation attempts were produced by seeding

in 25 cm^2 of Corning plastic flasks with 4 ml of cell suspensions containing 400,000 cells/ml in RPMI 1640 medium with 10% FCS.

2. Observed L. giganteum growing on tissue culture of Aedes albopictus (C6/36)

Flask cultures of Ae. albopictus (C6/36) mosquito tissue which had been subcultured 55 to 65 times were freshly prepared for infection by aseptic techniques. The flask cultures were incubated at 28 °C for 3 days. Then, the fungus was cultured in SFE agar for 3 days and cut in the proportion of 3/10 or 30% of area of petridish.and homogenized in 100 ml distilled water and homogenized at 5000 rpm for 30 seconds. For the test, the old media of C6/36 culture flasks were poured out and then 2 ml of the homogenate fungus were pipetted into the culture flasks. Then, 4 ml of new RPMI 1640 midium were added and incubated at 28 °C. After incubation for 3 days, the infected cell cultures were observed under the microscope for the fungal growth.

B. Preparation of fungal inoculum for infection

1. Culture preparation of L. giganteum

L. giganteum stock cultures grew on PYG agar and stored in the refrigerator. When subcultured agar blocks of fungal mycelium were transferred to fresh agar medium every 4 - 6 weeks for long term maintenance and cultured at 20 - 25 °C. The fungus was maintained both in liquid and solid medium.

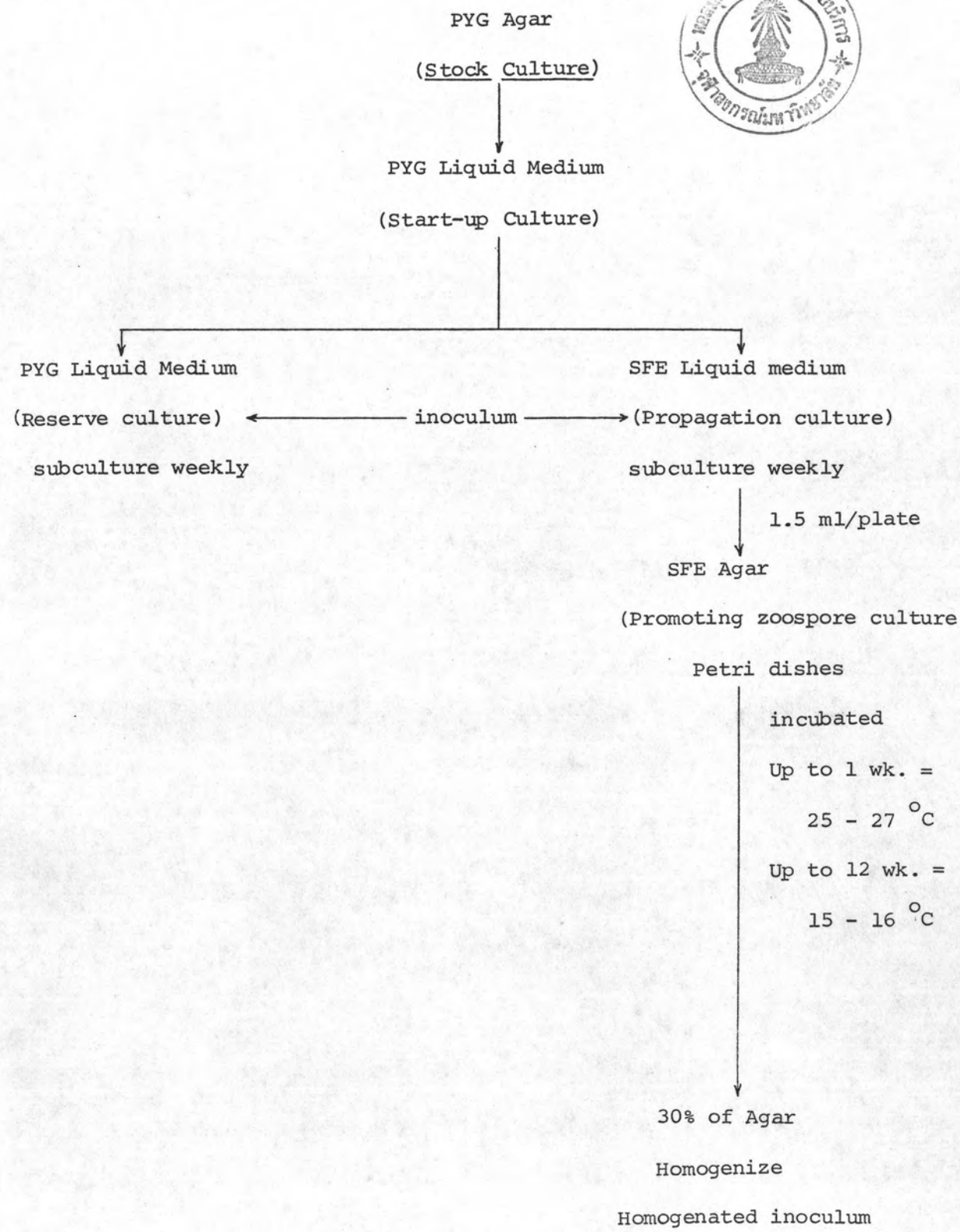


Diagram 3 Shows zoospore production of L. giganteum

2. Promoting growth and virulence zoospores of L. giganteum in liquid culture alternated with solid culture.

The procedure for this experiment followed Jaronski and Axtell (1984). They reported that the alternation of liquid and solid medium were effective in increasing zoospore formation in L. giganteum. Initial propagation of L. giganteum from stock cultures was carried out by (1.) in liquid PYG following Diagram 3. Five to six agar blocks were transfered to 100 ml culture medium PYG in a 250 ml flask and incubated at room temperature on a gyrotary shaker at 150 rpm for one week. The liquid PYG was kept as a reserve culture (Diagram 3) and used to restart SFE cultures when necessary.

After 1 week, 3 - 5 ml of liquid PYG culture were transfered to 150 ml of liquid SFE medium, (production culture in Diagram 3). In this medium the culture was recultured every week. Subsequent subcultures of SFE broth and PYG cultures were made weekly. All cultures were incubated at 25 - 30 ^{o}C.

After culturing L. giganteum for one week in liquid SFE medium, 3 - 5 ml were used to inoculate SFE solid medium and spread over the plate following the method in (3.). The inoculated liquid cultures of PYG and SFE were shaken gently on a gyrotary shaker at 150 rpm continuously.

The culture of L. giganteum in SFE broth was allowed to settle by placing the flasks in a slight angled position. The fluid was

poured out, washed with sterile water and replaced with 150 ml of sterile, deionized water. This washed culture was homogenized in a homogenizer at 5000 rpm for 15 seconds and inoculated on solid medium SFE agar.

3. Solid cultures of L. giganteum

Solid cultures of L. giganteum were used for promoting zoospore production. This method followed Jaronski et al. (1983). They found that the biphasic cultures of L. giganteum in SFE liquid medium (broth) and solid medium (agar) induced production of zoospores better than other media. For this experiment SFE agar was used. After incubating L. giganteum in SFE broth for 7 days, petri dishes of SFE agar were inoculated with 1.5 ml of the homogenate from (2.). Then the fungus were spread over the entire agar surface by gentle spreading and tilting the plate. Inoculated plates were incubated overnight and fungal cells were allowed to settle onto the agar and excess water was absorbed. The growing colonies developed and then the culture plates were kept in plastic bags and stored at 12 ^{o}C.

C. Studies on target organs of infection

Fifty, two-day-old mosquito larvae were placed in a beaker. The beaker contained 100 ml of homogenated inoculum, L. giganteum and 400 ml of distilled water and was incubated at room temperature. Three days later, some of the mosquito larvae were infected by L. giganteum. The dead larvae from the infection were studied by histopathological methods compared to normal larvae.

The steps of tissue preparation for microtechniques were done by taken the newly dead larvae and placing in a fixing agent. Three kinds of fixing agents were tried : 10% formalin, 70% ethyl alcohol and Bouin's solution.

The specimens were fixed overnight and kept in the metal cases. The cases were put in the auto technicon for dehydrating by ethanol in steps of 70%, 95% and 100%, then clearing by xylene, and impregnating in paraffin. The tissue was embedded in paraffin by placing on a mansoite rack. Each specimen was place in position with the appropriate identifying stirring tag.

The block cut by a microtome. The ribbons were placed on clean slides. Sections were drained approximately 1 minute before drying in a 60 ^{o}C oven for 30 minutes. Staining procedures were used Harris hematoxylin and Eosin (see appendix). After the slides dried, they were soaked in Harris hematoxylin for 15 minutes, rinsed in tap water, and differentiated in acid alcohol (see appendix), with three to ten quick dips. Slides were washed in tap water very briefly. The slides were then dipped in lithium carbonate water (see appendix), then washed in running tap water for 10 to 20 minutes and stained with eosin for 15 seconds. The dyed slides were dehydrated in 95% alcohol until excess eosin was removed, two changes of 2 minutes each, and checked under the microscope. Slides were dipped in absolute alcohol, two changes of 3 minutes each and then dipped again in xylene, two changes of 2 minutes each. The slides were mounted in Permount and microscopically examined.

D. Examined the fungus as cause of dead larvae

To ensure that the larvae died of the infection by L. giganteum; the fresh dead larvae were washed in sterile water for 2 minutes and then soaked in 0.5 g/100 ml penicillin solution for 2 minutes. Excess antibiotic was blotted on the sterile filter paper. The dead larvae were placed on PYG agar medium for 1 week. Examination of the fungus growth from the dead larvae was done under the microscope. The number of dead larvae were counted under the microscope.

E. The use of L. giganteum for controlling mosquito larvae

1. Study the virulence of L. giganteum to three species of mosquitoes.

The three species of mosquito larvae used in this experiment were Anopheles dirus, Aedes aegypti, and Culex quinquefasciatus.

The experiments were conducted in 600 ml. beakers containing 400 ml of deioninized water fifty two-day-old larvae were cultured and 1 - 2 tablets of food were added in each of fifteen containers. One hundred ml of homogenated L. giganteum (prepared from B.) were inoculated into nine beakers. The set of nine beakers consisted of three beakers containing 50 larvae of An. dirus, three beakers containing 50 larvae of Ae. aegypti, and the other three containing 50 larvae of Cx. quinquefasciatus. Three sets of two were used for controls without inoculum. The

experiments were conducted at room temperature, 28 ± 5 °C and a normal daylight cycle. Each beaker was covered with a sheet of cloth to protect it from dust and insects. The dead larvae were checked everyday and the data were recorded.

2. The susceptibility of An. dirus to L. giganteum

From the experiment E (1), the most susceptible species seem to be Ae. aegypti and Cx. quinquefasciatus. This experiment was made to confirmed the result from (1.) by testing only An. dirus as the test larva. The conditions of experiment were the same as (1.), excepted the amounts of water in the same size testing containers were less, in order to study the effects of the depth of water to the dispersion of zoospores.

In this experiment, six 600 ml beakers containing 400 ml of distilled water and50 larvae from two-day-old An. dirus and 1 - 2 food tablets were added. One hundred ml of fungal inoculum were inoculated into four containers which consisted of different levels of water and the other two were left as controls. The cultures were incubated at room temperature. The dead larvae were recorded everyday.

3. Effects of different parameters on infectivity of L. giganteum against Ae. aegypti

3.1 Selected types of medium to induce fungus zoospore formation

Five kinds of media were compared in order to increase the amount of zoospores of L. giganteum, by means of

zoosporangium formation. Selected media for growing the fungus were found by Domnas (1974, 1977, 1982), Jaronski and Axtell (1984). They were Peptone - Yeast extract - Glucose (PYG) from Domnas et al. (1974, 1977), Wheat germ - Yeast extract - Glucose (WGYG) and "Z" medium from Domnas et al. (1982), Hemp seed extract (HS) from Domnas et al. (1977), and Sunflower seed extract (SFE) from Jaronski and Axtell (1984). For testing, both broth and solid agar of each medium were used.

The preparation of fungal inoculum for media tests follows. The fungus was grown in PYG broth as previously described (also see appendix). PYG medium 100 ml in 250 ml Erlenmeyer flasks) was inoculated and incubated on a Gyrotary shaker for a week. Mycelia were collected and washed twice, resuspended to 100 ml. of distilled water and blended in the homogenizer at 5000 rpm for 20 seconds. Then, 5 ml was and inoculated into each of five kinds of test medium. The fungus was grown for 4 - 6 days until zoospores were detected. Then mycelium and zoospores were collected and washed twice with steriled water, resuspended in 100 ml with water and blended briefly for 5 seconds. One hundred ml of fungus was inoculated into 400 ml water in 600 ml beakers with 50 larvae of Aedes aegypti in each container. In each test, there were three treated beakers and one for control.

3.2 Zoospores induction by SFE and SFEA medium

Five kinds of media were compared for induction of zoospore production, i.e., PYG, PYGA, WGYG, WGYGA,

HS, HSA, Z, ZA, SFE, and SFEA, as showed in (3.1), the result from 3.1 showed that SFE and SFEA were the most effective media corresponding to active zoospore production. L. giganteum was grown on two phases of SFE medium, liquid and solid culture, the method following Jaronski (1984) procedures.

L. giganteum bioassays of the inoculum were conducted to ensure that the cultures were infective and viable by using twelve containers, each with 1 liter deionized water and 25 second - or third - stage mosquito larvae. The culture agar plates were cut at the rate of 78.0, 39.0, 3.2, 23.4, 19.5, 9.725 and 4.8625 cm^2 area of petri dish and then homogenated to suspension. Each of the ratios was inoculated into the container. One container of larvae was left as a control. The larvae were examined for infection after 48 - 72 hours.

3.3 Study on the inoculum size of L. giganteum and the percentage of mortality

The results from experiment 3.1 showed the best medium for production of effective zoospores by counting the percentage of mortality, and from the previous test (1.) Ae. aegypti was the most susceptible species. SFE and SFE agar were the selected media for cultivation of zoospores and the method for preparing the fungal inoculum was described earlier (3.1).

After culturing for 5 days on SFE agar, zoosporangium were formed. Divided the solid culture of SFE agar at the rate of 4.8625, 9.725, 19.5, 23.4, 31.2, 39.0, 78.0 cm^2 of the area of agar culture petri dish. Each part of the culture was

blended separately in 100 ml of distilled water in the homogenizer. The suspension was poured into 250 ml Erlenmeyer flasks, continuously shaking at 150 rpm. for 12 hours. While waiting for zoospore production, 50 two-day-old larvae of Ae. aegypti were counted and put into 600 ml beakers containing 400 ml of water. Twelve hours later, L. giganteum from different concentrations were added to each beaker. Another two additional beakers of water containing 50 larvae of Ae. aegypti were set as control. The bioassays were conducted at room temperature. After 72 hours, the larvae were examined for infection by the fungus.

3.4 Study the larval age versus susceptibility

To investigate the effect of age of Ae. aegypti larvae on their susceptibility to L. giganteum, 23.4 cm^2 cultivated agar was cut from the cultured plate and blended as described above, dispensed into 400 ml of water in 600 ml beakers. Larvae of Aedes aegypti were selected, and grouped according to age starting from egg stage. Hatch times from eggs were designed as follows : first-hatch, second-hatch (larvae emerging in the first 24 hr period), third-hatch. To obtain larvae of different ages, larvae were maintained in rearing trays from hatch until the desired age was reached. The larvae were then transferred to treatment beakers containing 400 ml of distilled water in which 50 larvae per beaker were placed. Percentages of infection and mortality were recorded. The records were done 72 hours after inoculation of the fungus. Infection of larvae was confirmed by microscopic examination and proved by culturing the infected larvae individually on growth media. Uninoculated controls were maintained in the experiments.

3.5 Effects of acidity (pH) of cultured water on infection rates

To investigate the effectiveness of zoospores infecting the mosquito larvae at various acidities (pH) of water, the fungus was grown on SFE for a week and prepared as an inoculum following the method of Jaronski (1984) and Domnas (1977) as described. A cut at a rate of 23.4 cm^2 area of fungal cultured on SFE plate was blended in a homogenizer with 100 ml of distilled water and continuously shaken at 150 rpm for 12 hours. After that, 100 ml of the suspension was poured into the beakers along with 2-day-old larvae of Ae. aegypti and 400 ml of distilled water with various pHs. This test was assayed for infectivity at various pHs of cultured water by counting the percentage of larvae mortality. The selected acidities for this study were set as 5.6, 6.0, 6.7, 7.0 and 8.2.

3.6 Effects of temperature of water on L. giganteum infectivity

Inoculum were prepared as described above. The treated experiment and the control were set. Six different temperatures were set 12, 20, 25, 28, 30 and 37 ^{o}C. The percentages of dead larvae were recorded and presented as mortality rates.

3.7 Effects of cultured water from different habitats on L. giganteum infection to Ae. aegypti larvae

To investigate the affects of various habitats of cultured water on zoospore infection, the fungus was grown for

5 days on SFE agar and then 5 days in SFE agar. Mycelium and zoospores from SFE agar were prepared as described earlier. Water was taken from six different habitats : Klong Mahanak, Slum, high tide water from Chao Praya river at Sathu-Pradid, low tide water from Chao Praya river at Sathu-Pradid, pond water with fountain aeration in front of Chulalongkorn University, pond water without aeration in Chulalongkorn University, and rain-water from the reservoir. The fungus suspensions of 100 ml were inoculated into 400 ml of sampling water and incubated at room temperature. The results were recorded after 72 hours.

3.8 Effects of Biological Oxygen Demand (BOD and Chemical Oxygen Demand (COD) on the infection of L. giganteum ot Ae. aegypti larvae

Effects of BOD and COD on zoospore infection in the larvae were determined. The inoculum was prepared as described in B. Then 100 ml of inoculum were dispensed into 400 ml of water from different places which had different BODS and CODS. Each treatment had three replications, and one without fungus was used as a control. BOD and COD measurements followed the Standard Method (1971).