CHAPTER 4

DISCUSSION

Cultures of L. giganteum in mosquito cell lines

For studying the culture of L. giganteum in a mosquito cell line, the Ae. albopictus cell line (C6/36) was used for the purpose of fungal propagation and to increase the pathogenicity by reinfection of the host cells. Singh and Paul (1969) have shown that Ae. albopictus cells were more sensitive than other cells for isolating dengus viruses from human sera. In 1971, Chappell et al., (1971) also noted that this line was superior to infant mice for the isolation of dengue virus from mosquitoes during the 1969 epidemic in Puerto Rico. For this study, continuous mosquito cell lines for fungus propagation of L. giganteum were used. The following results of infection were seen: the fungus infected the mosquito cells, and mycelial of L. giganteum grew in the invertebrate cell culture.

The results obtained indicated that L. giganteum made the cells slough off from the surface of the culture flask and some of them became smaller and degenerated. No zoosporangium were produced. Domnas et al. (1977), Elliot et al. (1978) indicated that L. giganteum was unable to synthesize sterols and required exogenous sterols before it produced zoospores. Dean and Domnas (1983) found that L. giganteum produced extracellular protease (trypsin-like) which required a suitable inducer such as protein. They also reported that the use of specific substrates on inhibitors could demonstrate the presence of

collagenase, a trypsin-like protease and a weak elastase from the fungus. These enzymes can digest the living host cells.

McInnis (1974) demonstrated transferase and hydrolase (β-glucosidase) activity while screening the organism for its enzymatic capabilities. Cellobiase was detected but not very rapidly. McInnis and Domnas (1974) also noted that the in vivo function of intracellular aryl-β-glucosidases in fungi was not clear; however, extracellular forms could release glucose from aromatic glucosides for subsequent uptake and catabolism. The function of L. giganteum β-glucosidase was that if it was located at or on the cell surface, it could then hydrolysed aryl-β-glucosides present in the host insect circulatory system. It then released glucose for fungal uptake and at the same time released toxic aglycones into the insect cells. These enzymes and toxic aglycones could cause the cells to die. Fungus propagation in invertebrate cell culture, showed that L. giganteum was unable to complete its life cycle.

Studies of target organs of mosquitoes for infection

As a facultative parasite, L. giganteum can grow vegetatively either as a parasite of mosquito larvae or as a saprophyte in the aquatic environment, where it apparently prefers a littoral habitat (Willoughby, 1969). The life cycle in Diagram 1 shows both asexual and sexual reproduction reported by Couch (1935), Couch and Romney (1973), Domnas et al. (1974), McCray et al. (1973 a b), Umphlett (1973), and Umphett and Huang (1972). The parasitic phase was initiated by a laterally biflagellate zoospore. The authors found that the

zoospores could encysts on the cuticle, and also on the pharynx, spiracles, anal papillae and posterior abdominal segments. The fungus penetrated the cuticle to the haemocoel. Federici (1981) reported that the trypsin-like enzyme aided in the penetration. After the haemocoel was completely filled with mycelium, vegetative growth ceases and reproduction begins. Domnas et al. (1974) indicated that larval death was most likely the result of starvation. At this stage each hyphal segment becomes asexual spores. In asexual reproduction, sporangia, the rounded zoosporangiums were formed from a discharge tube which passed through the cuticle. The terminal hyaline vesicles were differentiated within 24 hours into biflagellate zoospores. The vesicles ruptured and zoospores infected mosquito larvae or other suitable substrates.

Microscopic observations of larvae exposed to the fungal inoculum revealed that L. giganteum produced zoospores and oospores in the larva, especially in the head and digestive tract (Fig. 10, 11, 12, 13). The mycelial growth most frequently occurred in the head area and grew towards the interior with infection at the anal segment and gills (Fig. 15, 16). These observations might suggest that some of motile zoospores were ingested and probably entered the alimentary track, germinating inside the tissues of the host larva through the anterior portion of the digestive tract. The hyphae appeared to grow via the haemocoel and spread to the anal segment and gills within three days after inoculation. The hyphae spread throughout the body. L. giganteum could produced zoospores and oospores inside the tissue of the larva as shown in the Fig. 10, 11, 12, 13, 14, 15, 16. The cases of zoosporangium also were seen, showing that L. giganteum

produced zoosporangium and released motile zoospores within three days. This shows that L. giganteum could be an effective mosquito control agent.

McCray et al. (1973) demonstrated infection of motile zoospores through the mouth and direct penetration through the exoskeleton. Umphlett and Huang (1972) indicated that this occurred within 72 hours after infection, where the coelomic cavity of the larva was filled with mycelial growth, and in many instances hyphae could be seen growing in the aortae of the larvae. Death of the larva occurred in about 24 hours.

Jaronski and Axtell (1984) studied the target organs for infection by L. giganteum and found evidence of infection in the head region initially. Subsequently, they saw proliferation in the thorax which became filled with mycelia, zoosporangia and dark-colored spherical oospores.

Examination the fungus as a pathogen

To ascertain that the fungus caused infection and death, the dead larvae were cultured on PYG agar. The results are shown in Fig. 16, 17, 18.

Fig. 16 showed that the fungal mycelium had penetrated outside the siphon of the infected larvae. The mycelium seen were smaller than in the cultured media. Microscopic examination of the infection showed the fungal mycelium to be rod-shaped and small. (Fig. 17). Oospores were also seen (Fig. 18). These results indicated that this fungus changed forms after penetrating inside the larva. This

observation is supported by McCray et al. (1973) and Jaronski and Axtell (1984).

Determined the susceptible species of mosquito larvae to infection

In Fig. 19 the mortality rate is shown; 6.0, 67.3 and 70.0% for An. dirus, Ae. aegypti and Cx. quinquefasciatus, respectively. These data indicate that L. giganteum is an effective pathogen for culicine mosquitoes.

The dead larvae of all species were examined microscopically throughout the course of the tests. All were found infected and fungal development was the same in all of the susceptible species. Mortality occurred within approximately the same time as found by Umphlett and Huang (1972). No difference was observed in the appearance of the hyphae or zoosporangium. The fungus developed and produced motile zoospores in all infected specimens.

The results presented here are comparable to earlier findings which indicated that in subsequent isolations in North Carolina experiments, L. giganteum infected only Culicidae. Another North Carolina isolate of L. giganteum was equally infective to both culicine and anopheline larvae (McCray, 1985). Results shown in Fig. 19 point out that this imported strain infected both culicine and anopheline larvae but at different percentages of mortality. However, Jaronski (1982) found that Anopheles were not susceptible. McCray et al. (1973) studied the susceptibility mosquito species to L. giganteum and found that except for the anophelines, all species became infected. On the other hand, L. giganteum (ATCC 36942) was able to infect Anopheles sp. but at a low percentage of infection. Although there are conflicting

reports about the infectivity of L. giganteum for anophelines, mosquito species susceptible to isolates of L. giganteum were tested by Federici (1981). He showed that in general the fungus has a broad host range, particularly against culicines.

The effects of the depth of water to the infection by zoospores of L. giganteum

The percentage of mortality of An. dirus was considerably less than that of either the Culex or the Aedes mosquitoes (Fig. 19). Ramoska et al. (1982) indicated that the mortality rate increased because either Cx. quinquefasciatus and Ae. aegypti had more chances of contacting zoospores of L. giganteum. Increasing numbers of zoospores were consumed by the larvae beneath the surface with decreasing depth of the water. In order to ascertain if any mortality could be attributed to the hypothesis that with constant numbers of zoospores, does the depth of water affect the percentage of infection. The experiment was designed by decreasing water depth and incresing the zoospore density.

The depth-response curve is as shown in Fig. 20 howing that the larvae contacted the zoospores. The infectivity of zoospores at various depths of water was checked after 72 hours (Fig. 20). The results showed that An. dirus did exhibit a little higher mortality in the 100 ml. of water at 1.8 cm. of depth than it did for the higher depths.

There are some reports on habits of Anopheles species. McCray et al. (1973 a, b) indicated that the feeding habits of anopheline larvae caused an obvious reduction in the infection rate

of populations of An. albimanus, An. quadrimaculatus, An. stephensi, and An. sundaicus. He found that when the larvae fed, the portal of entry was restricted by culicular penetration rather than through the double portals of buccal cavity and cuticle. They observed the anophelines routinely grazed at the bottom of the container rather than the surface film.

Ramoska et al. (1982) discussed mortality of An. dirus larvae versus its habits. It is a surface feeder while both Cx. quinquefasciatus and Ae. aegypti feed below the surface.

Umphlett and Huang (1972) noted that L. giganteum was not effective against Anopheles spp. Since larvae feed and rest on the surface of the water. These habits restricted severely the number of contacts made between larvae and zoospores.

Effect of media on zoospore production

An infective unit of L. giganteum was a biflagellate zoospore (Jaronski, 1982), this experiment examined the media that promoted the production of zoospores. Suitable media for large scale production of zoospores of L. giganteum have been described by Domnas et al. (1982) and Jaronski et al. (1983), i.e., WGYG, HS, Z medium were counted. The results from Fig. 21 showed that L. giganteum which was cultured on SFE and SFE agar medium produced many more zoospores than the others. Zoospore production by L. giganteum following culture in biphasic culture systems i.e., in liquid phase and solid phase was $2.5 \times 10^5 - 6.0 \times 10^6$ zoospores/ml. The fungus cultured on PYG, PYGA, WGYG, WGYGA, HS, HSA, Z, ZA media produced 182, 70, 250, 197, 412, 220, 500, 775 zoospores/ml., respectively. PYG and WGYG broth and agar were

very poor substrates for zoospore production as determined by counting the percentage of mortality. The numbers obtained from "Z" medium were good but not better than SFE. When either WGYG or HS medium was used alone in the liquid phase of culture, larval mortality was much lower than when these two liquid media were combined as "Z" medium. When PYG was used for the liquid phase, larval mortality was very low.

Domnas et al. (1977) indicated that L. giganteum did not pooduce infective zoospores when grown on a simple undefined medium such as PYG. Zoosporogenesis was induced either by growth on aqueous extracts of hemp or soy beans, or on sterol - free media which had been supplemented with sterols or mevalonic acid.

Warner et al. (1983), Lord and Roberts (1985) found that L. giganteum required exogenous sterols for formation of zoospores. Elliot et al. (1978), Haskins et al. (1977) and Hendrix (1980) noted that L. gigantuem was unable to synthesize sterols and required exogenous sterols for sexual and asexual reproduction. Kerwin and Washino (1983), however, suggested that the fungus retained its ability for zoosporogenesis, even following prolonged maintenance on sterol-free medium.

Domnas et al. (1976) indicated that zoospore production by L. giganteum was induced by growing the fungus on hemp seeds or whole hemp seed agar. In their study an attempt was made to identity the sterols involved in zoospore formation.

In the studies of Boswell et al. (1977), methods of inducing zoosporogenesis of L. giganteum were studied. Results showed that L. giganteum had an absolute requirement for sterols for zoosporogenesis.

Jaronski and Axtell (1984) determined the range of nutrient concentration in SFE suitable for zoospore induction and compatible with the production system. Soluble protein concentration was used as an indicator of nutriednt concentration in FSE.

Effect of different growth periods on zoospore production

The use of L. giganteum for biological control of mosquitoes requires a simple and rapid means of mass-producing infective zoospores. The ability of L. giganteum to produce viable zoospores and infect mosquito larvae under laboratory conditions was determined by bioassays using larvae of Ae. aegypti.

For assays of zoospore production, the isolate of L. giganteum was cultured following methods similar to those used by Jaronski et al. (1984), L. giganteum was grown in SFE broth for 1 week, then transferred to SFE agar. The fungus in each container was harvested every day at the same time. Each day, the area of 23.4 cm^2 of the SFE agar plate was cut and blended in an homogenizer with 100 ml distilled water. Zoospore counts were begun 12 hours post immersion and determined by direct counts in a haemacytometer. Inspection of the agar has been described by Domnas et al. (1982). Zoospore counts obtained from SFE cultures every day are shown in Fig. 22 and indicate that culturing on SFE agar for 5 days produces more zoospores than other methods. The highest number of approximately 6.0×10^6 zoospores per ml was obtained (Fig. 22). The level of zoospore production was reproducible in SFE medium. Peak zoospore production was unrelated to the relative level of zoospore and the period of inoculum. On the sixth day and seventh day, the number of zoospores dropped. This was because of the deficiency

of sterol in the media for promoting zoospore formation. The counting method counted only the zoospores but not the mycelia and the old zoospores might have already germinated.

Domnas et al. (1982) indicated that considerable variation in many experiments was encountered, with identical mycelial preparations and yielded zoospore numbers ranging from zero to 40,000/ml. One reason for the variation might be that the zoospores gather in the upper surface layer of the container. Prior to this observation, the cultures were swirled to obtain a uniform distribution of zoospores per unit volume. However, swirling led to a loss in flagella and a decrease in motile zoospores. In several experiments, zoospore counts dropped drastically in tubes that had been gently swirled and zoospores without flagella were seen.

The results from the experiments of Domnas et al. (1982) indicated that the cultures grown for 4 or 5 days produced the most zoospores. Best zoospore production was obtained when the organism was first grown for 4 days in PYG, followed by growth on Z medium for 4 - 6 days; the number of zoospores was 14,667 - 36,367 zoospores/ml. However, considerable variation was encountered in these experiments.

McCray (1985) studied Ae. aegypti, Ae. taeniorhynchus and Ae. sollicitans. Infection and fungal development occurred in the same manner as in Culex and produced death of all individuals at approximately the same time.

Effect of inoculum sizes on infection rates

The purpose of these experiments was to minimized the size of inoculum with mixed zoospores and mycelium. Two-day-old larvae were exposed to the inoculum. The inoculum was tested prior to experiments to ensure that the cultures were infective and viable.

The results shown in Fig. 23 demonstrated that the best inoculum size was 3/10 unit of inoculum or 23.4 cm^2 of SFE agar plate incubated for 5 days. This provided 6.0×10^6 zoospores/ml. The results showed that more than 65% of the mosquito larvae were killed. If the inoculum size was increased to 39.0% the mortality rate increased to 86%. This was the smallest unit of inoculum that killed more than 50%. However, it was found that the range of 23 - 39% of inoculum worked well.

Effect of mosquito age on infection

Various larval stages were studied. The period of time from hatching of eggs to emergence of adults was 11 days, 1.e., from egg to the first instar took 1 day, from first instar to fourth instar took 7 days, from fourth instar to the pupal stage took 2 days, and from pupa to adult took 1 day. The larval age was studied relation to infection. A diagram of mosquito stages is shown in Diagram 2.

Figure 24 summerizes the results using only Ae. aegypti. This experiment determined the ages that were susceptible to infection. It showed that larvae of Ae. aegypti were the most susceptible to infection by L. giganteum. In early experiments in this study, 2-day-old larvae were used because Umphlett and Huang (1972), Domnas et al.

(1974) and Jaronski (1984) said that this age was highly susceptible to infection at all dose levels used. A decreasing effectiveness of the pathogen was noted as larval age increased (Fig. 24). One to Two-day-old larvae were killed by L. giganteum at a 90% level. Examination at the third instar larvae in Fig. 10, 11, 12 revealed that the fungus penetrated through the larvae and caused death within three days. If the first instar larvae were tested, infected larvae occurred in the third instar. The few infected larvae still alive when counted succumbed during the next 2 - 3 days. Larvae escaping from the infection usually pupated and emerged if permitted to continued development. Only a few infected pupae were seen. This is supported by McInnis (1974) when he found that the motile zoospore encysted on the chitinous exoskeleton and penetrated the cuticle with a germ tube The pupae were rarely infected because of the thickness of the exoskeleton. The second reason was that the feeding behaviour of larvae at different stages might be different; the young larvae fed at the bottom while the old larvae fed in a lower depth of water. The third reason was that in the fourth instar larvae, the larvae pupated and did not feed on anything. It might be possible that as fourth instar, the larvae fed less than younger larval stages, so the chance of the fungus going inside the larva was also low. The last reason was that the buccal apparatus of the fourth instar had developed completely and the fungus was screened out.

McCray et al. (1973) also studied host-age versus infection and found that all larvae of Ae. aegypti and Cx. quinquefasciatus that were early third instar or younger when exposed became infected and died without pupating. Some late third instars occasionally pupated. Early fourth instars when infected frequently pupated and

died as pupae. Late fourth instars when exposed usually did not become infected and pupated and emerged as healthy, uninfected adults. No infected adult has been found in this study. Those adults produced by larvae that were exposed as late fourth instars failed to induce infection when placed in with test larvae.

Effects of acidity on infection

Laboratory experiments were conducted to determine the pH range of water medium for infecting larvae. The larvae used in this experiment were Ae. aegypti; 50 2-day-old larvae per container and an inoculum size of 3/10 units.

Mosquito larvae were infected in the pH range of 6.0 to 8.2 (Fig. 25). The infection rate of L. giganteum at pH 6.7 and 7.5 was higher than at other pHs.

Axtell et al. (1982) demonstrated that the fungus infected mosquitoes in a pH range of 6.3 - 8.9 in both laboratory and field experiments. Lord and Roberts (1985) noted that L. giganteum poorly tolerated extremes of pH range at pH 4.5 and 8.0, but he showed that the infection occurred at pH 8.2 which was higher than what Axtell et al. (1982) found.

Effect of temperature on the infection

The effects of various temperatures on the infection rates of L. giganteum (Fig. 26) showed optimal temperatures for infection ranged between 20 to 28 ^{o}C. When the temperature was higher than 30 ^{o}C, infection was not observed. Larvae infection was high at 20 - 28 ^{o}C,

but decreasing infection rates occurred below 20 °C and above 28 °C . The results showed that at temperature below 15 °C and the higher than 37 °C the mosquito larvae died both control and treated larvae. This temperature range corresponds to the range for zoosporogenesis reported by Jaronski et al. in 1982.

Jaronski and Axtell (1983) found that high larval infection rates occurred in range of 21 - 29 °C for two strains of L. giganteum.

McCray (1985) found that at temperatures near 38 °C and below 16 °C, the fungus was non-infective. Temperatures around 38 °C interfered with infection by the zoospores and also killed them. Low temperatures around 16 °C also interfered with infection.

Effect of BOD, COD and different types of water on infection by L. gigantuem

The objective of this study was to determine the BOD and COD range of water samples from different locations and their effect on L. giganteum growth and infection of mosquitoes. Different habitat effects with certain mosquito species was determined.

The results in Fig. 27 showed BOD and COD measurements of various types of water (Klong Mahanak opposite National Railway station),slum at Klong Toey, high tide and low tide from Chao Praya river at Sathupradid, pond water in Chulalongkorn University, pond water with aeration in front of Chulalongkorn University, and rain water in the reservoir). High tide water and rain water had the lowest BOD and COD which were 3.63 mg/l and 2.407 mg/l for BOD and 16.0 mg/l and 12.936 mg/l COD (Fig. 27). The others were between

9.786 mg/l and 60.3 mg/l for BOD and 50.6 mg/l and 200.80 mg/l for COD, respectively. The pHs of the various types of water were between 6.06 and 8.45. The pHs of high tide water and rain water were close to the optimal pH (6.7) for infection (shown in Fig. 25).

Fig. 28 and Fig. 29 show that the percentages of mortality of mosquito larvae depended upon different BODs and CODs. Larval infections by mycelial cultures of L. giganteum resulted in 20 to 90% mortality for inoculated beakers and 0 to 64% for control beakers. The tests were performed in several series, each representing dilutions from a particular water source. Within each series, the highest pollution level gave 3% infectivity, and the lowest gave 83.5% infectivity. As dissolved oxygen in the water decreased, the concentrations of BOD and COD increased (Fig. 28, 29). The ability of L. giganteum to infect mosquito larvae decreased in relation to the percentage of infection in their respective controls. The results from Fig. 25 showed that high tide water and rain water (3.63 mg/l and 2.407 mg/l BOD) killed more larvae. The high tide water and rain water were also good for infection (Fig. 29), in which the amounts of COD were 12.936 mg/l and 16.0 mg/l, respectively.

High tide water and rain water were good for the promoting of infection of L. giganteum in the presented species (Fig. 27, 28 and 29). Normal habitats of Ae. aegypti are still and unpolluted water where the amount of BOD is not beyonded 20 mg/l. From these studies, the results showed that several environmental variables in the target sites affected the infection of L. giganteum i.e., water pH, BOD and COD of the water. These results are supported by Jaronski and Axtell (1984) who reported that the fungus was likely to be effective in

relatively unpolluted water habitats such as flooded plain pools, ditches, and irrigation systems. Jaronski and Axtell (1982) also said that no infection occurred in water with low to moderate levels of pollution similar to the results in Fig. 27, 28 and 29. The infection rates measured against BOD and COD concentrations revealed that L. giganteum had little tolerance for organic pollution as supported by Axtell et al. (1982), Jaronski and Axtell (1982) and Lord and Robert (1985).