CHAPTER 3

RESULTS

Cultures of *L. giganteum* in the mosquito cell line

The initial culture of *Aedes albopictus* cell line (C6/36) was obtained from Armed Force Research Institute of Medical Science (AFRIMS), and maintained in RPMI 1640 plus 10% foetal calf serum. After subculturing for 3 days, several patches of attached cell were seen and many pieces were adhering to the container surface (Fig. 1). Cells were growing over all the surface area of the culture flask. Microscopical examination of the tissue of this line in RPMI 1640 media showed predominantly epithelial cells with a small proportion of fibroblastic cells. Although the major part of the cell sheet was a monolayer, cell aggregates were seen regularly in the cultures (Fig. 2).

After inoculating *L. giganteum* into the C6/36 culture for 3 days, the propagation, the infection stage and pathogenicity of pathogen were studied. The cells which were stuck to the flask's surface began to float, and the number of cells decreased. Upon examination of the cells through the inverted microscope, the infected cells were observed to become smaller and to grow around *L. giganteum* mycelium (Fig. 3). Group of cells (syncytia) coalesced and began to slough off from the surface, compared to the cells in the control flask which looked normal. The day after, almost all the syncytia had detached from the surface (Fig. 4) and tended to be smaller and

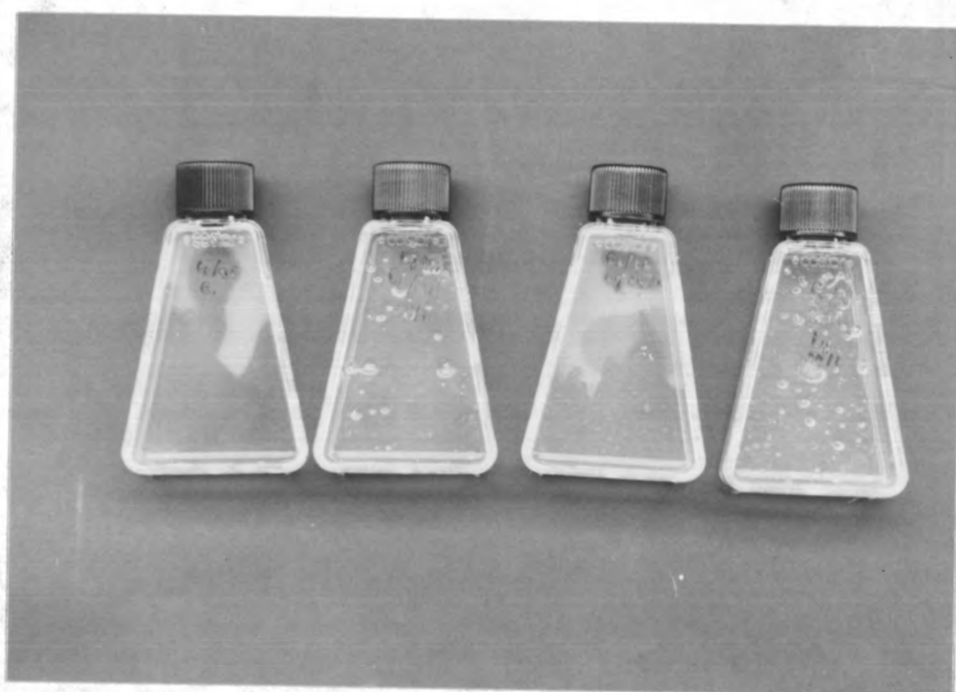

Fig. 1 Growth of Lagenidium giganteum on the mosquito cell line (C6/36) after 3 days.

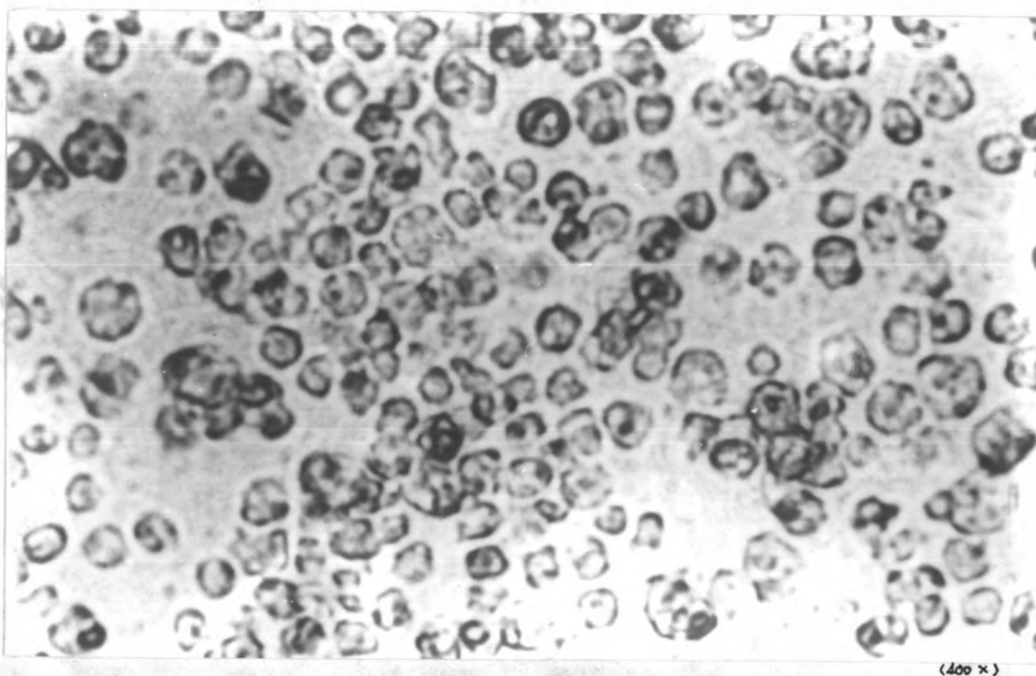

Fig. 2 Growth of the normal cell line (C6/36) after incubation for 3 days.

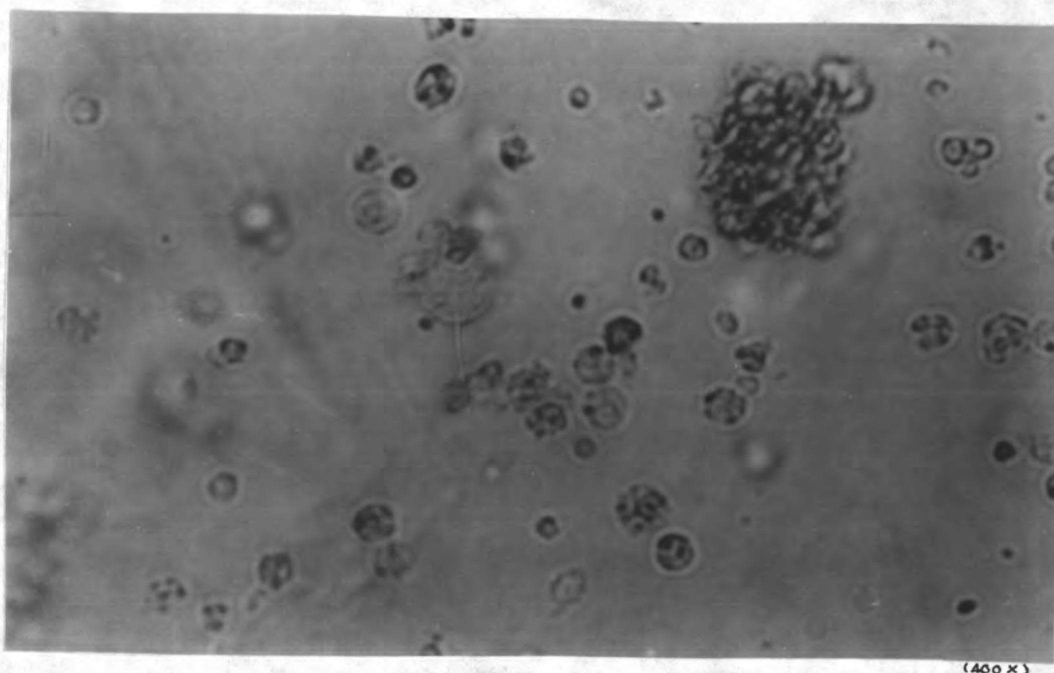

Fig. 3 Growth of the infected cells with clump of L. giganteum after inoculation for 3 days. The density of cells were decreased.

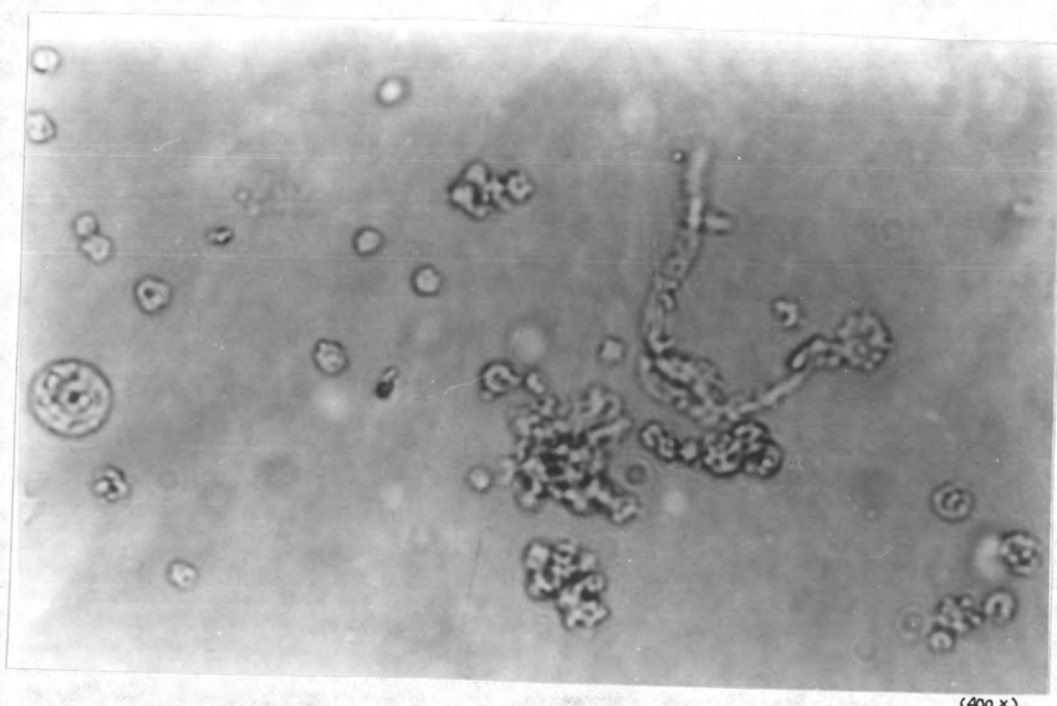

Fig. 4 Mycelium of L. giganteum growing after inoculation into the mosquito cell line. Cell debris was seen around the fungal mycelium.

degenerate. The cell layer in the infected cultured appeared thinner compared to the control. Extensive mycelial growth was seen however, no zoosporangium were formed.

Fungal inoculum preparation for infection

The method used for the fungal inoculum followed Jaronski and Axtell (1984). L. giganteum was cultured first in PYG broth medium, then was transferred to SFE broth medium for inducing zoospore formation. After 1 week in SFE broth medium, fungus was transferred into SFE agar medium. Two or three days later, zoospores were seen when examined under the microscope. The results are shown in Fig. 5 and Fig. 6. Fungus which was cultured in PYG medium showed irregularly branched or unbranched mycelium, but mycelium in SFE medium looked smaller than in PYG medium (Fig. 5, 6). Zoospores (Fig. 7, 8) were reinform, broadly fusiform or grape - seed like, with flagellates.

Microscopic study of target organs with infection

The infected larvae were examined and studied in sections of tissue. Selected suitable fixatives for tissues were tested. Three kinds of fixing agents were tested i.e., 10% formalin, 70% ethyl alchohol and Bouin's solution. The results showed that 70% ethyl alchohol was the best fixative for fixing the infected mosquito larvae.

The infected larvae, dead for not more than 24 hours, were fixed in 70% ethanol overnight, then dehydrated by alcohol, cleared by xylene, impregnated with paraffin and embedded in the auto technicon embedding for 12 hours. After embedding with paraffin, the

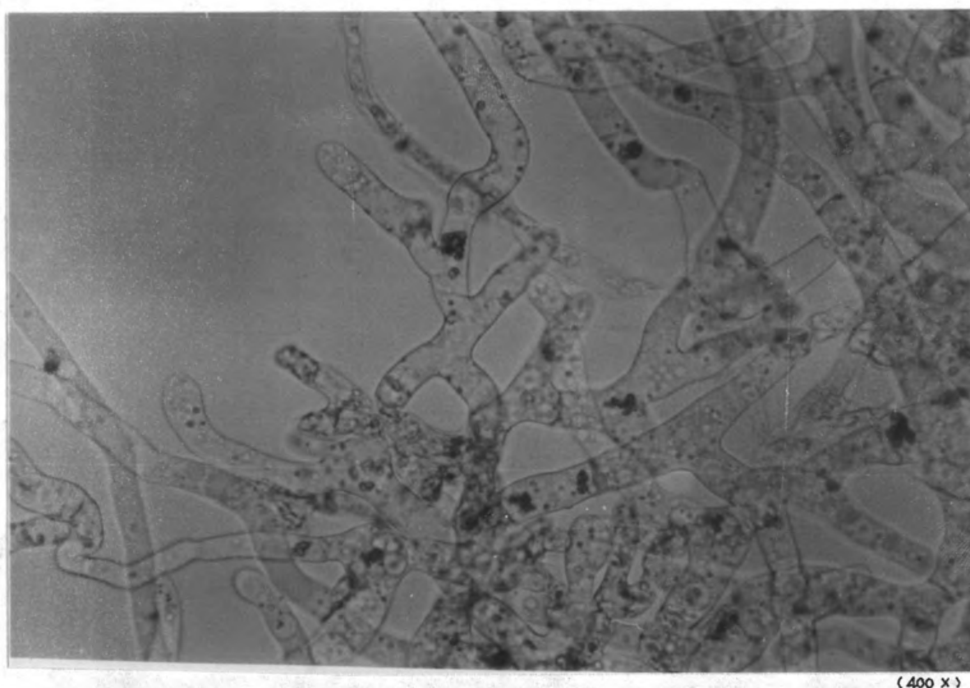

Fig. 5 Aseptate mycelium of L. giganteum growing on PYG medium.

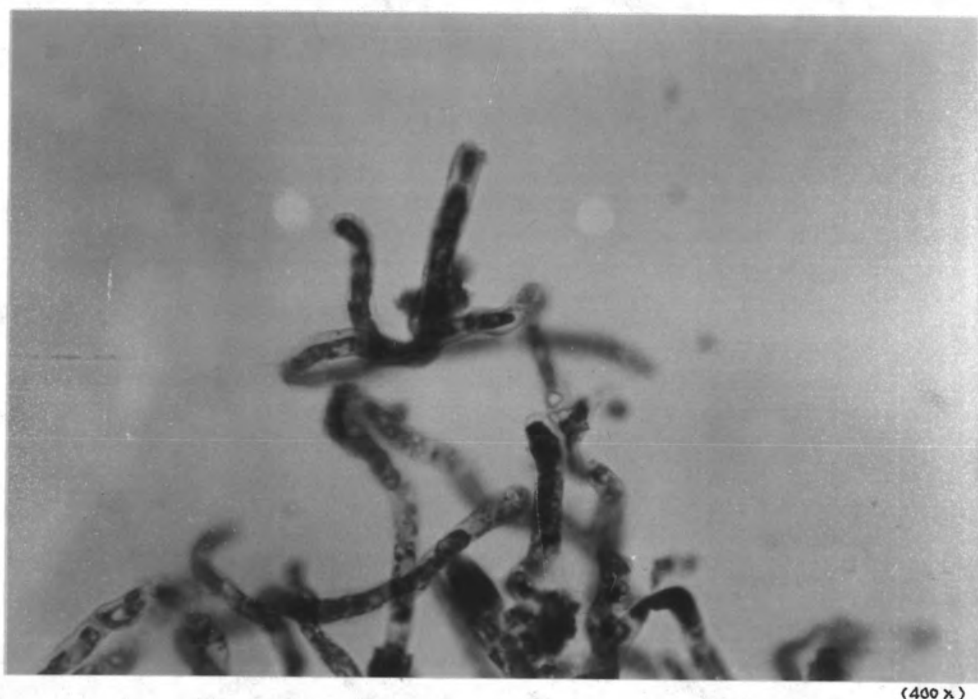

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Fig. 6 Mycelium of L. giganteum cultured in SFE broth medium. The density of the cytoplasm was increased when compared the culture in PYG.

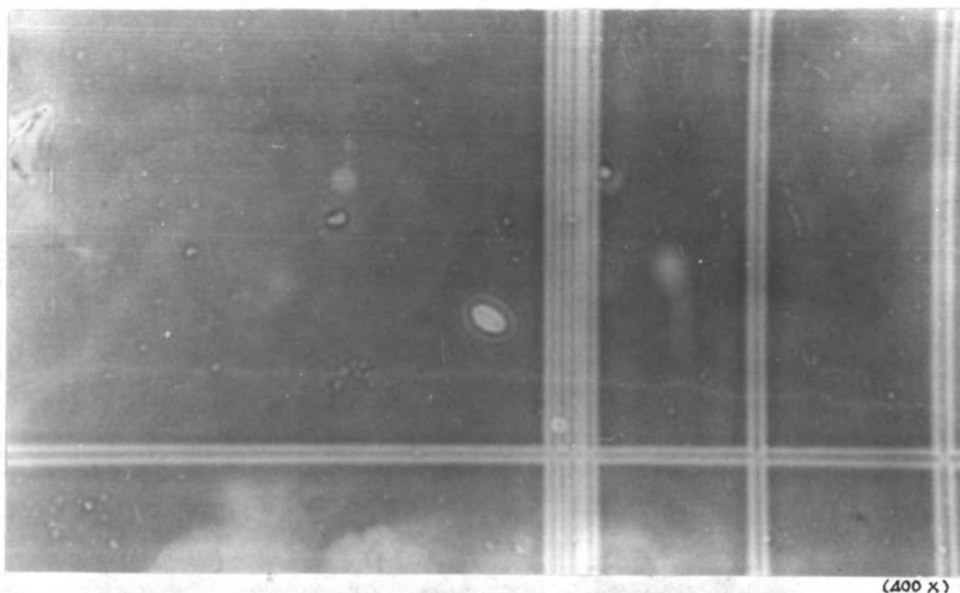

Fig. 7 Kidney shape of secondary zoospores.

Fig. 8 Germination of zoospores of L. giganteum in cultured water.

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Fig. 9 Long-section of normal larva when stained with hematoxylin and Eosin.

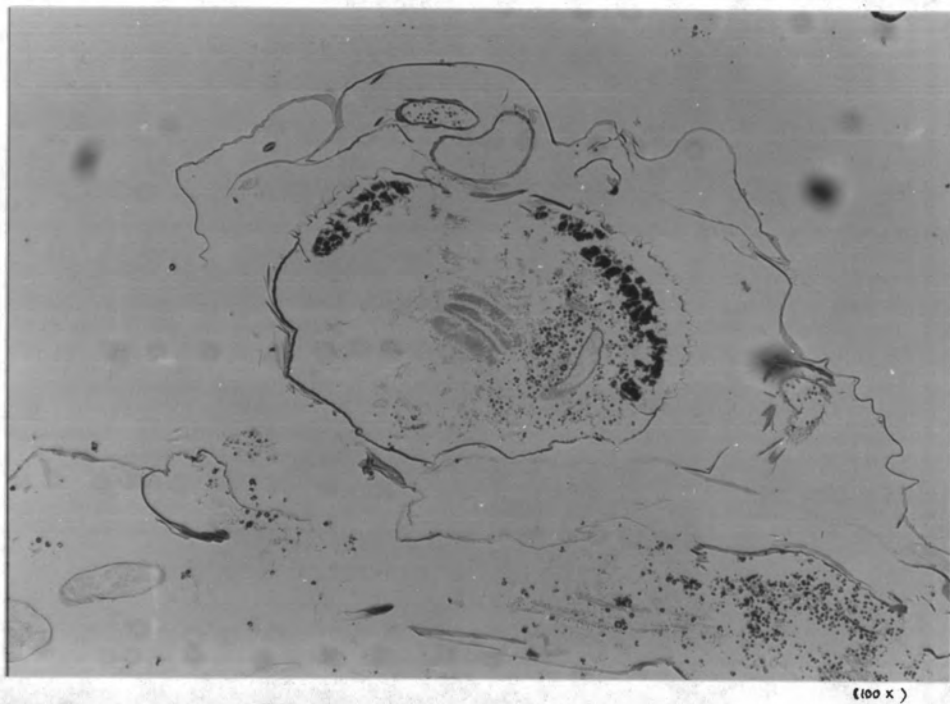

Fig. 10 Fungal infection at the head region of the infected larva

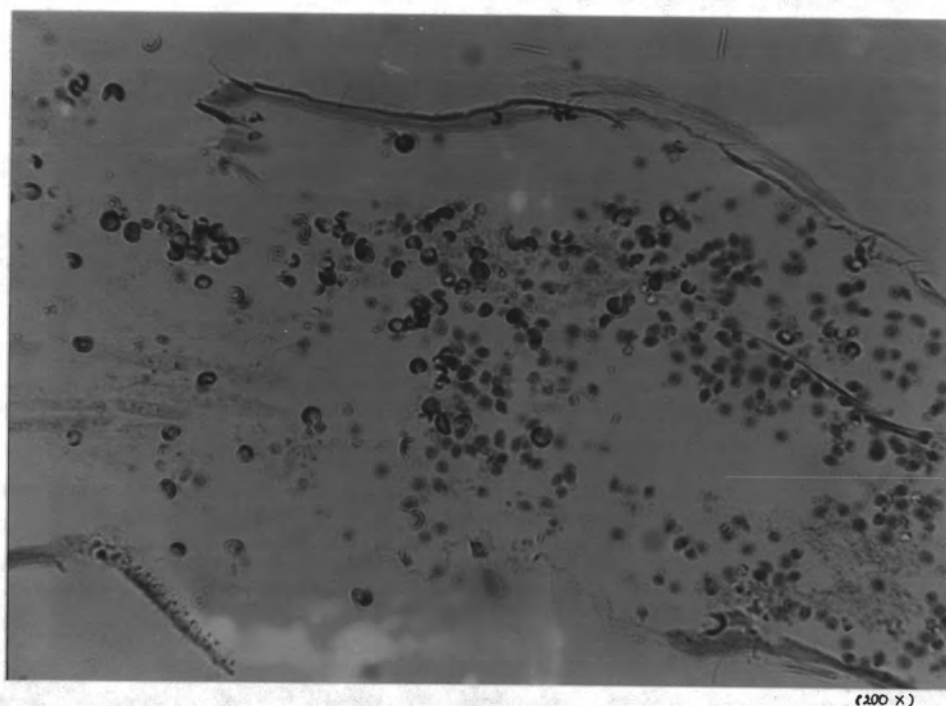

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Fig 11 Fungal infection at the thorax region of the infected larva

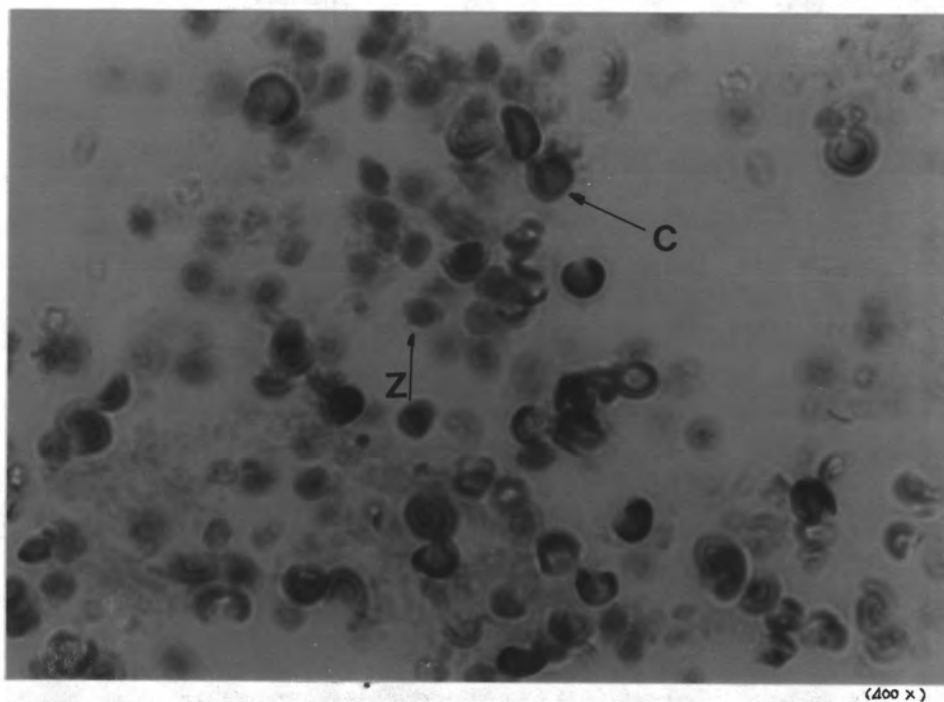

Fig. 12 The zoospores of L. giganteum inside the mosquito larva. The walls of zoosporangium were seen.

C = case of zoosporangium

Z = zoospore

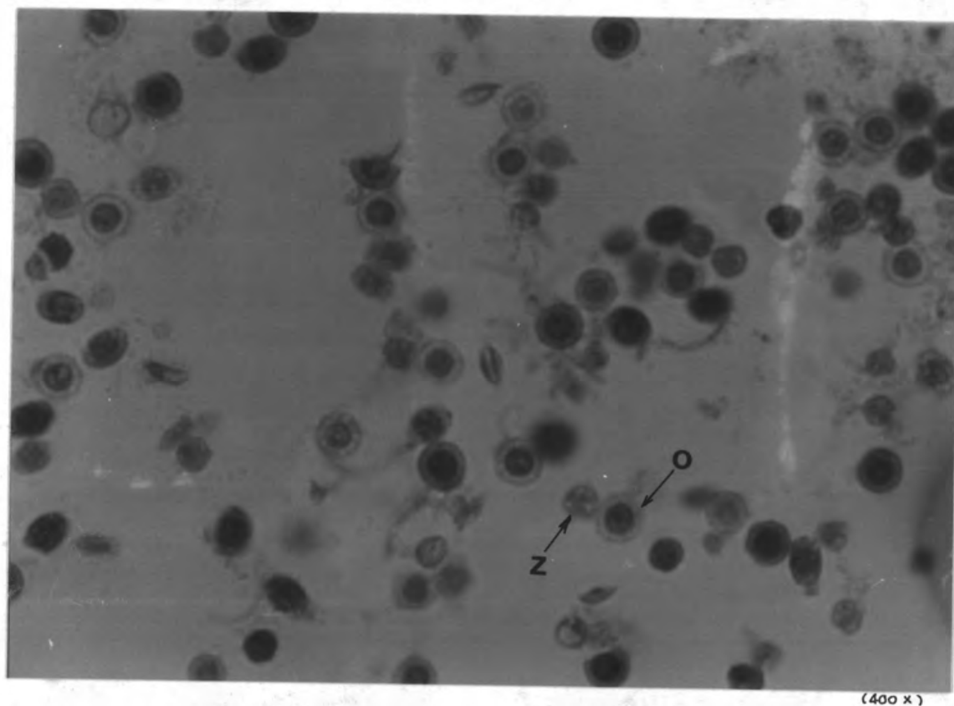

Fig. 13 Zoospore and Oospore formation inside the infected larva

O = Oospore

Z = Zoosporangium

tissues were cut 5 μm thick by a microtome. The techniques of Harris, hematoxylin and Eosin were used for staining slides.

Microscopic examination of the normal (Fig. 9) mosquito larva compared to an infected (Fig. 10, Fig 11) ones showed that the whole bodies structure was not affected much but the fungal mycelium were seen inside the organs of the infected larva, especially in head, thorax and the internal organs such as the midgut and hind gut (Fig. 10, Fig 11). L. giganteum produced both zoospores and oospores inside the larva (Fig. 12, Fig. 13). Cases of zoosporangium were also found. With whole mount, the fungal mycelium and zoospore formation were seen (Fig. 15) compared to the normal (Fig. 14).

Examination of the fungus as a pathogen

The infected larvae were tested to determine that the fungal infection caused death.

Collected dead infected larvae were washed in distilled water and soaked in Penicillin solution 0.5 ppm for 2 - 3 minutes. Dead larvae were blotted and cultured on PYG agar for 4 days. The results showed that the mycelium were growing around the dead larvae. The larvae with fungal growth were counted as cause of death by fungal infection. As seen by microscopic examination, the mycelium appeared around the larva and also inside the larva itself (Fig. 16, 17, 18).

Determinatiuon of susceptible mosquito species to L. giganteum

Fifty larvae of each species, Aedes aegypti, Anopheles dirus and Culex quinquefasciatus, were counted and used for exposure to

Fig. 14 Normal digestive tract, top view of the mosquito larva.

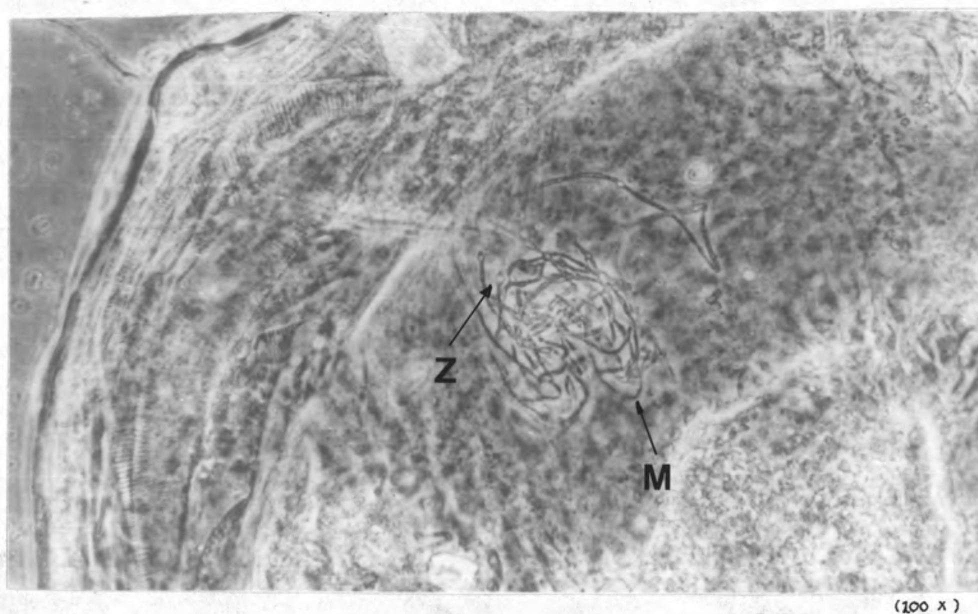

Fig. 15 Digestive tract of an infected larva. The mycelium were growing inside, and zoospores were seen at the hypha terminal.

M = Mycelium

Z = Zoosporangium

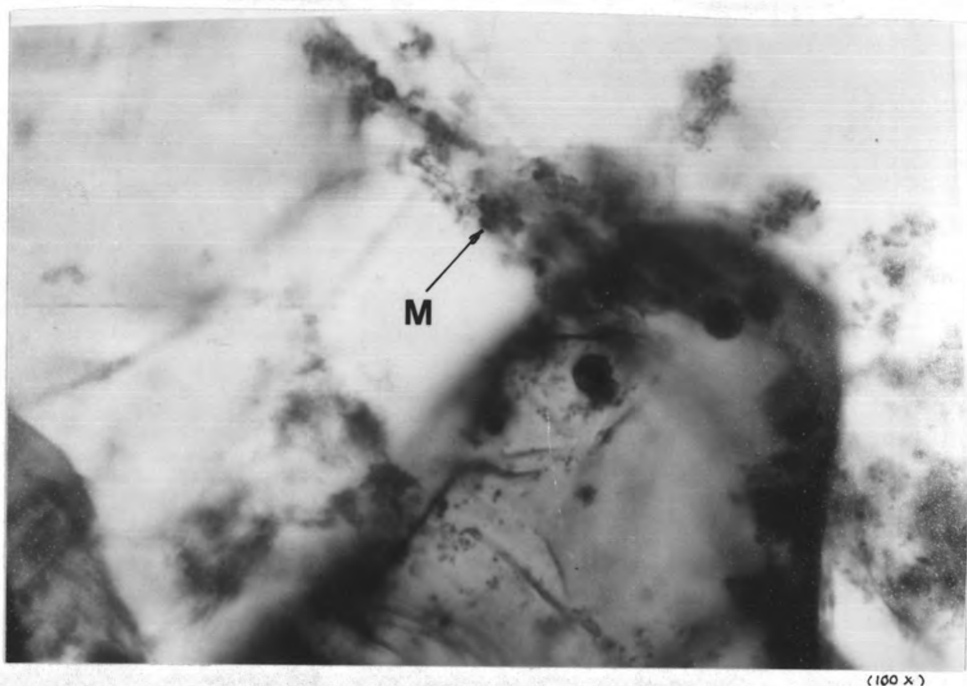

Fig. 16 Mycelium of L. giganteum protruded out around the siphon of an infected Ae. aegypti larva (whole mount)

M = Mycelium

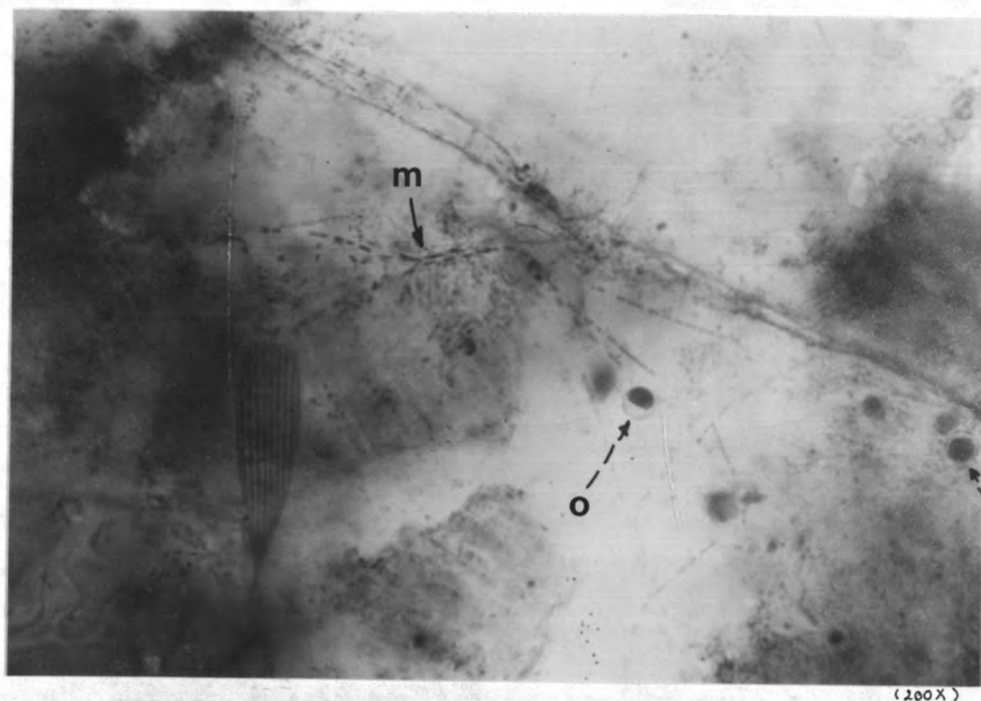

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Fig. 17 Mycelium of L. giganteum infected Ae. aegypti larvae (whole mount).

M = Mycelium

O = Oospore

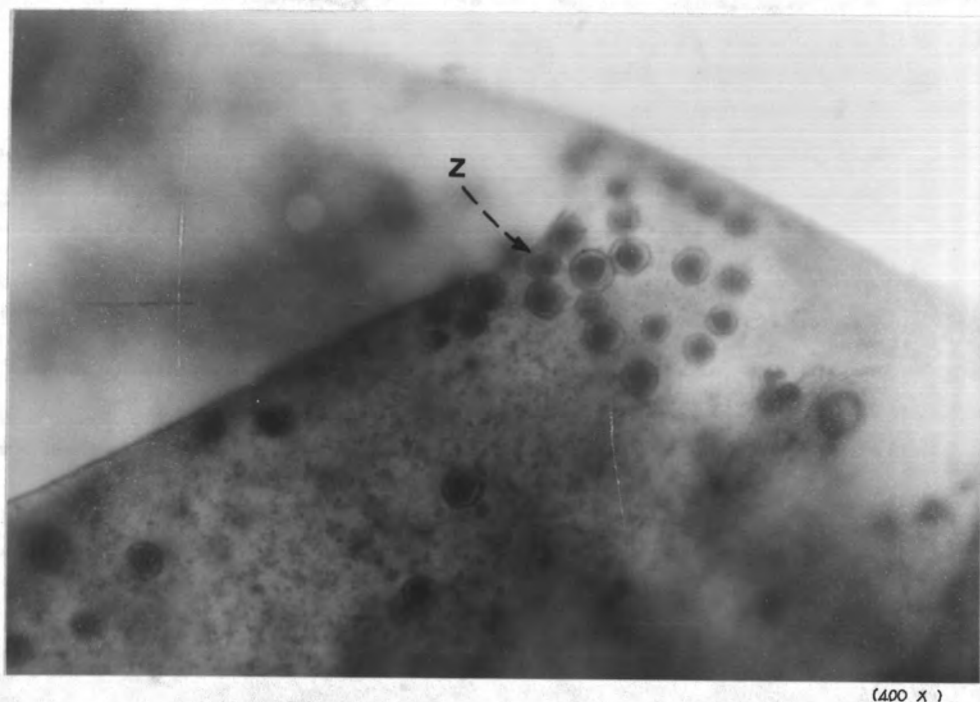

Fig. 18 Zoospores of L. giganteum spread over the siphon of an infected Ae. aegypti larva.

Z = Zoosporangium

L. giganteum using the technique of Jaronski (1984) by which the fungus was grown on SFE medium in order to produce zoospores, under laboratory conditions. Fifteen beakers containing 50, 2-day-old larvae were inoculated with 10 ml of homogenized inoculum in SFE medium. The larvae were observed for the infection at 72 hours after inoculating the fungus. The data was recorded at 3 day intervals until the larvae became pupae and then adults. The results were shown in Fig. 19. The infection rate was fecorded as a percentage of the mortality rate.

The results showed that the infection level in An. dirus was low. Only 6.0% of the larvae died, as compared to 67.3% and 70.0% with Ae. aegypti and Cx. quinquefasciatus, respectively, which were killed by L. giganteum.

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

The results shown in Fig. 19 indicate that Ae. aegypti and Cx. quinquefasciatus larvae were killed by zoospores of L. giganteum more readily than the larvae of An. dirus at the conditions with 9.0 cm. depth of water. The infection rate at various depths of water were studied to determine the effects of the depth of water to infection of An. dirus by the L. giganteum. Due to the dispersion of zoospores in large amount of water, the chance of infection might be difficult. The experiment was set for An. dirus to test that the depth of water influences the chance of infection. The same size of fungal inoculum from 10 ml from SFE culture was inoculated in various volumes of water; 100 ml, 300 ml, and 500 ml of water with depths of

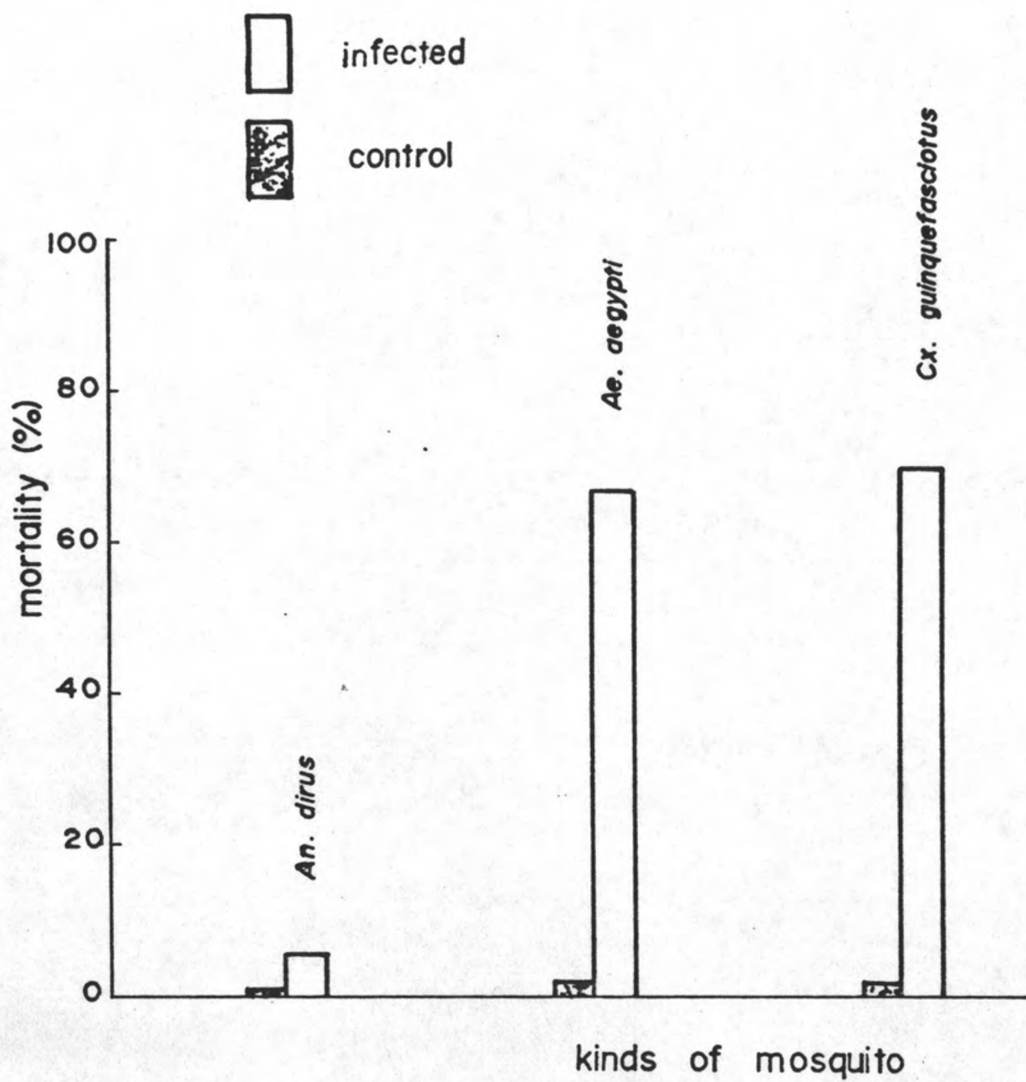

Fig. 19 Percentage of mortality of three species of mosquitoes infected by L. giganteum

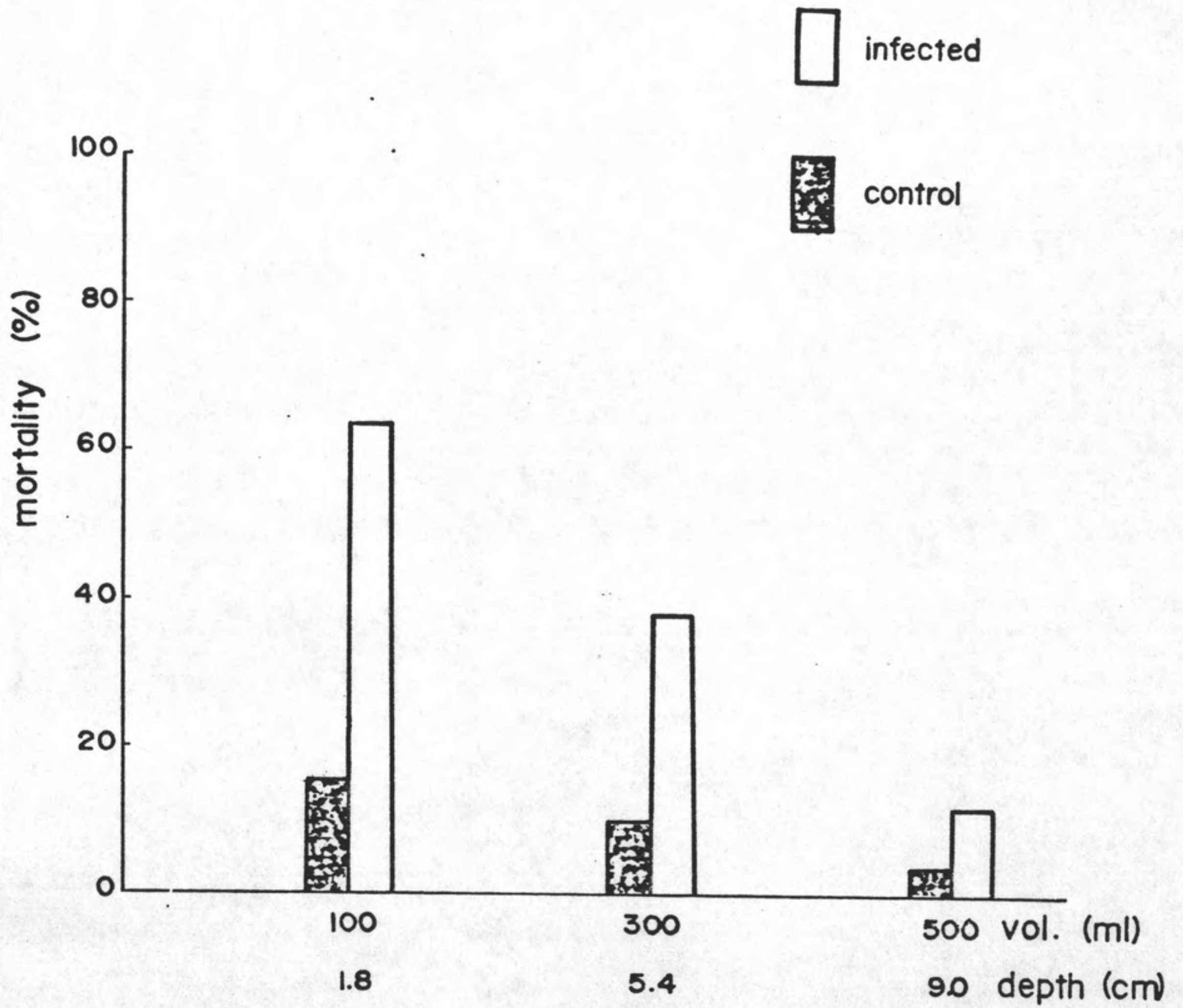

Fig. 20 Percentage of mortality of An. dirus after infection by L. giganteum at various depths of water.

1.8 cm, 5.4 cm., and 9.0 cm., respectively. The results were recorded and presented in Fig. 20 and showed that the percentage of mortality in 100 ml, 300 ml, and 500 ml of water were 64.0%, 38.0% and 12.0%, respectively. Some mosquito larvae died in control sets, i.e., 17.0% mortality in 1.8 cm depth of water, 10.0% in 5.4 cm depth of water and 4.0% in 9.0 cm depth of water.

Effects of media on promotion of zoospore production

The media which were used in this test were prepared following Domnas et al. (1974) and Jaronski (1984), and were PYG, PYGA, HS, HSA, WGYG, WGYGA, Z, ZA, SFE and SFEA.

Data presented in Fig. 21 to show the best medium for zoospores production was obtained by measuring the percentage of mortality of 2-day-old *Ae. aegypti* larvae. The percentages of mortality of the larvae were 8.67, 4.00, 22.67, 10.67, 15.33, 8.00, 35.33, 38.00, 66.00 and 76.00% when *L. giganteum* was cultured in Peptone-Yeast extract-Glucose (PYG), PYG agar (PYGA), Hempseed extract (HS), HS agar (HSA), Wheat germ-Yeast extract-Glucose (WGYG), WGYG agar (WGYGA), "Z" medium (Z), Z agar (ZA), Sunflower seed extract (SFE), and SFE agar (SFEA), respectively. From these results, the best medium for promoting zoospores production was SFE medium both liquid and solid medium.

Effective period of zoospores for infection

The amount of zoospores were couned by haemacytometer after culturing *L. giganteum* in SFE broth for 1 week and then transferring to SFE agar. The cultures were checked for contamination, and microscopically observed for zoosporangium production. The increasing

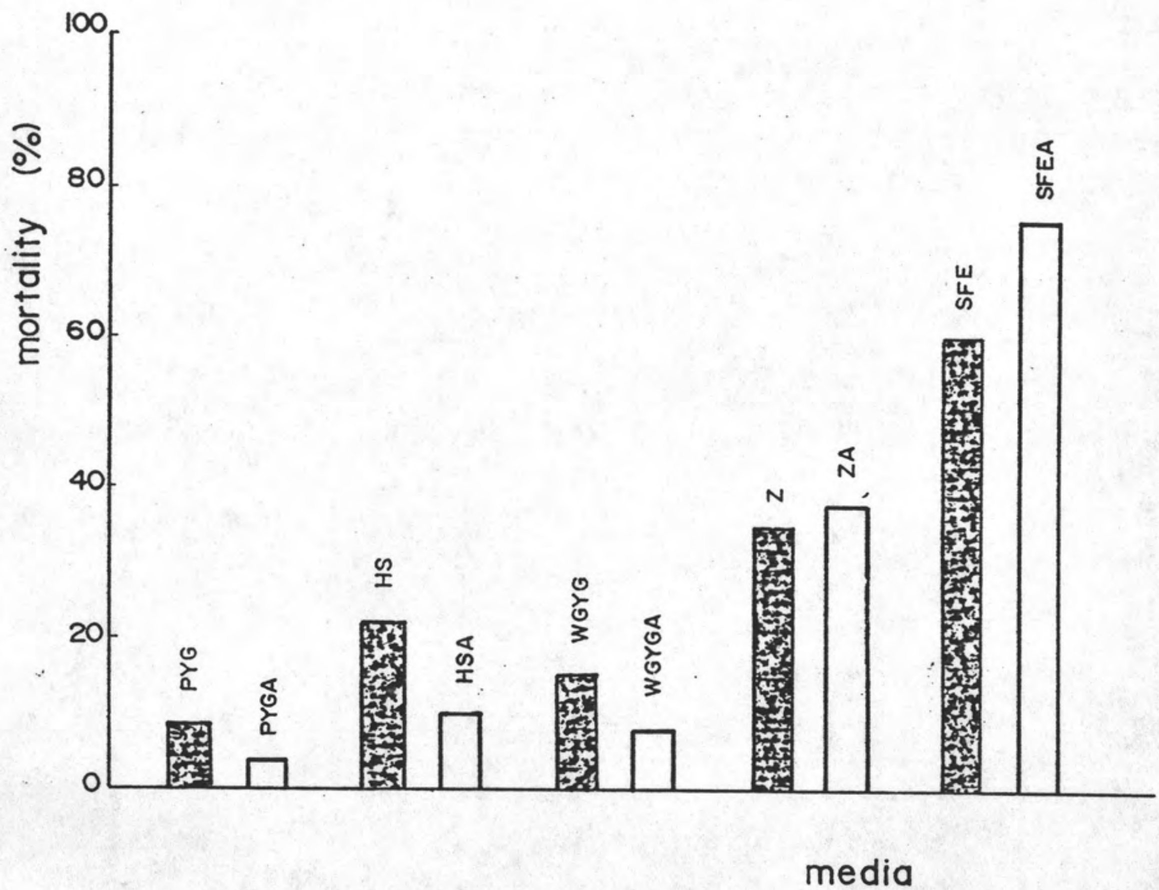

Fig. 21 Percentage of mortality of Ae. aegypti larvae after infection of L. giganteum which was cultured on various media.

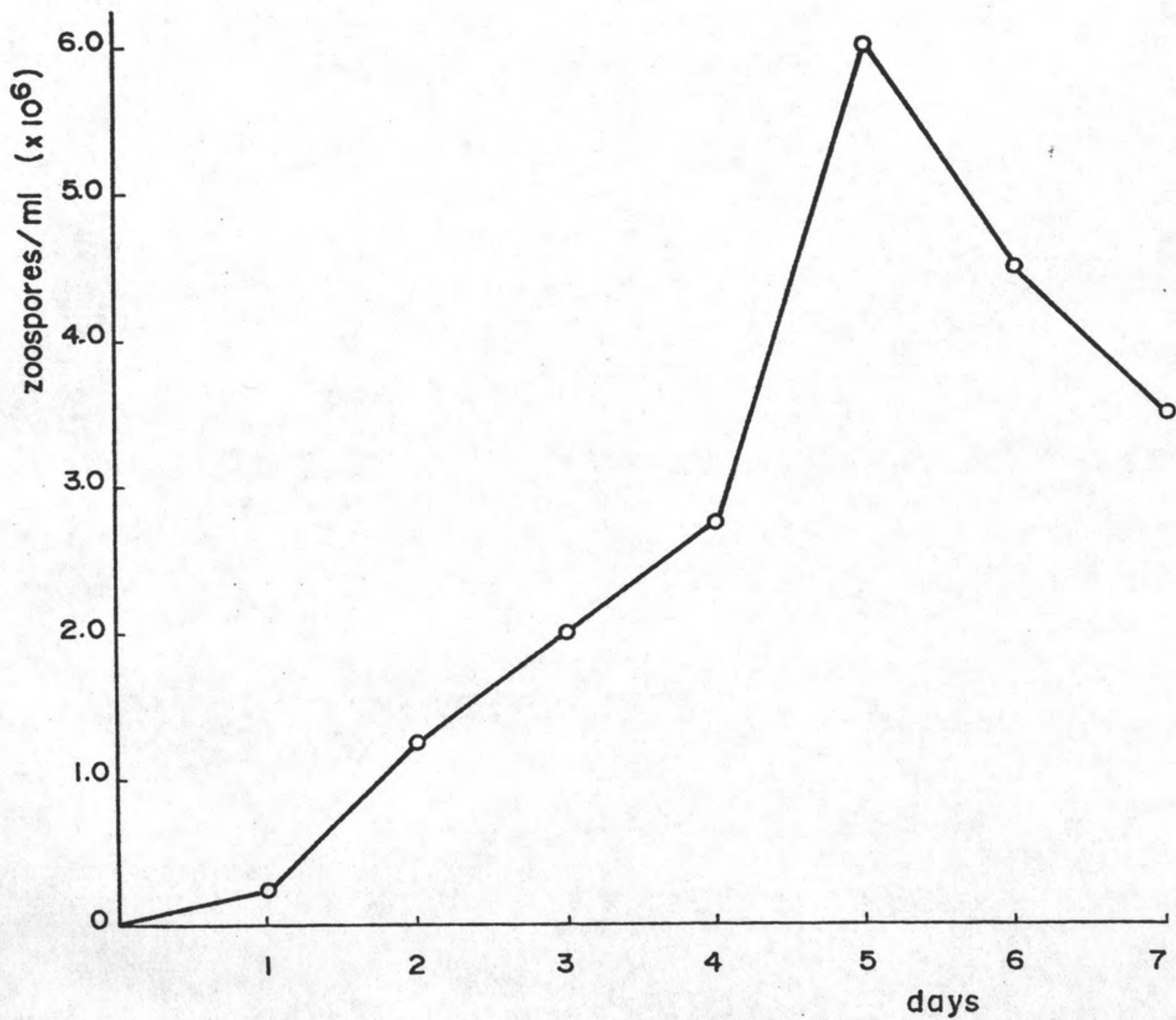

Fig. 22 Number of zoospores of L. giganteum per ml after incubation for various numbers of days.

amount of zoosporangiums implied an increasing amount of zoospores. The suspension of zoospores from SFE agar were counted everyday by a haemacytometer under the microscope. The results of this study are shown in Fig. 22. The amount of zoospores per ml of the inoculum were 2.5×10^5, 1.25×10^6, 2.00×10^6, 2.75×10^6, 6.0×10^6, 4.5×10^6 and 3.5×10^6 when the periods of incubations were 1, 2, 3, 4, 5, 6 and 7 days, respectively.

Effect of inoculum size on the infection rates

The experiment yielding the data presented in Fig. 23 was designed to determine whether or not greater inoculum size were required to obtain favorable kill levels in large populations of larvae of *Ae. aegypti*.

Concurrent with, or prior to, laboratory introduction of *L. giganteum*, bioassays of the inoculum were conducted to ensure that the cultures were infective and viable. This assay consisted of several containers, each with 1 liter deionized water and 25 second- or third-instar mosquito larvae. A suspension of homogenized *L. giganteum* agar culture was added to each container at the rate of 1/2, 1/4, 1/8, 1/16 petri dish of culture per container. After 72 hours, the larvae were examined for infection.

In this test, twenty-four containers were set in four groups with 50, 2-day-old *Ae. aegypti* larvae in each container. The containers in each group then received 78.0, 39.0, 31.2, 23.4, 19.5, 9.75 and 4.875 cm^2 area of inoculum. One area of inoculum was *L. giganteum* which was cultured in a petri dish of SFE agar for 5 days. The results for this test shown in Fig. 23 were presented as the percentage

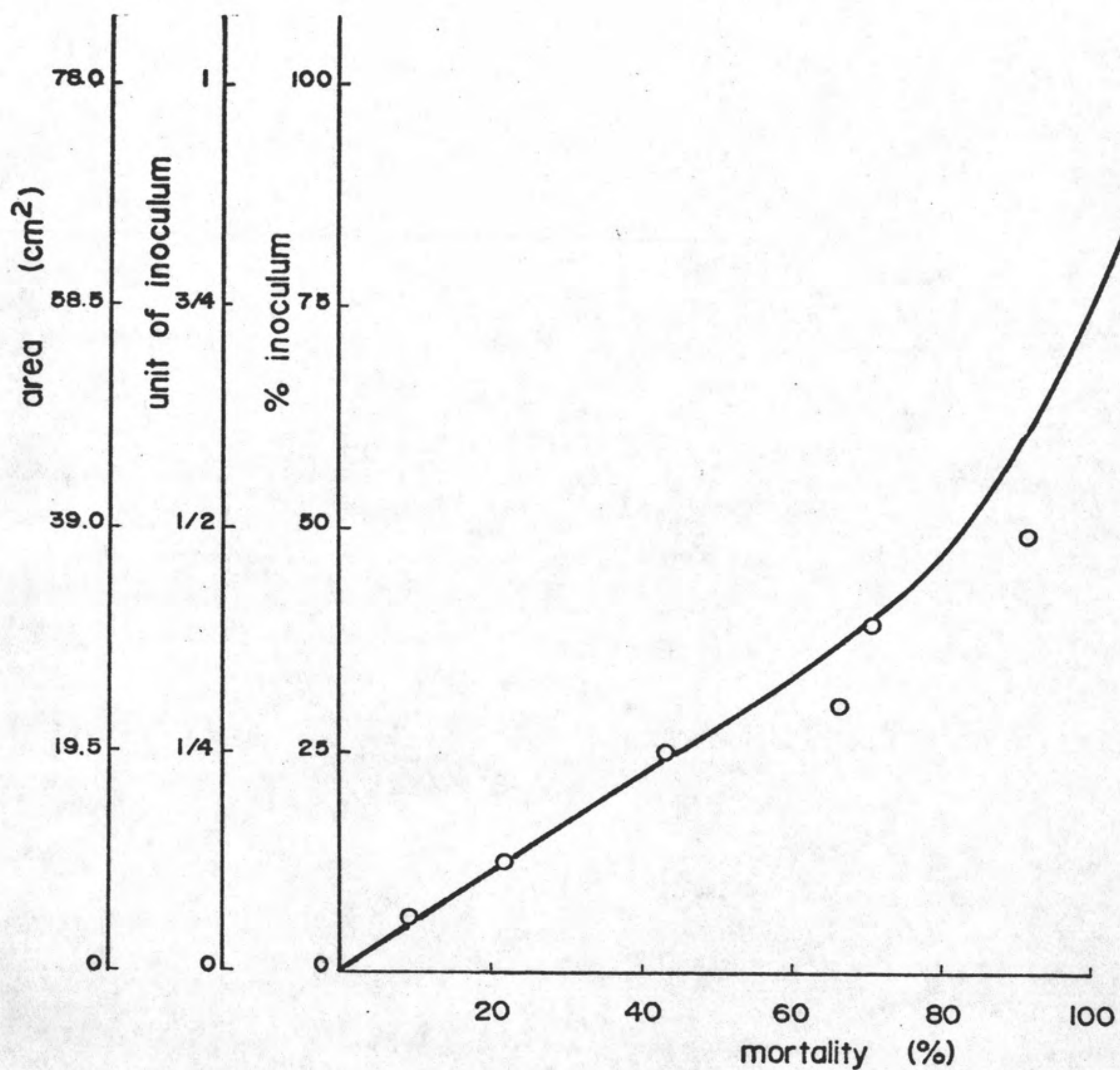

Fig. 23 Percentage of mortality of <u>Ae. aegypti</u> which were inoculated with various percentages of inoculum for 5 days.

of mortality of the larvae for each size of inoculum above, i.e., 100, 92.0, 70.67, 66.67, 43.33, 22.0, and 9.33% of mortality for 78.0, 39.0, 31.2, 23.4, 19.5, 9.75 and 4.875 cm^2 area of inoculum, respectively. These results show that the inoculum sizes of fungus which is good for infection was 23.4 cm^2 area of inoculum.

Effects of mosquito larval age on zoospore infection

Figure 24 shows the larval aged which were susceptible to the infection of *L. giganteum*. The larvae tested were of varying ages, and the time of the infection counts were made at 72 hours after inoculation. With 23.4 cm^2 area of inoculum, the percentages of mortality were 96.67, 94.67, 80.0, 42.67, 20.67, 18.67 and 13.33% for the larval ages at 1, 2, 3, 4, 5, 6 and 7 days old, respectively. These results show that early instar larvae (about 1 - 2 days after hatching were most susceptible to the infection of *L. giganteum*.

Effect of acidity of water on zoospore infection

Investigation on the effects of acidity of water on zoospore infection was done when *L. giganteum* cultured on SFE agar for 5 days and treated as described in Fig. 23. The results of pH experiments are shown in Fig. 25. The percentages of mortality of infection were 54, 76.67, 88.67, 84.67, 81.33 and 94% when the pH levels were 5.6, 6.0, 6.7, 7.0, 7.5 and 8.2, respectively. The normal larvae in the control were also and had 46, 50, 22, 34, 20 and 46% of mortality at pH levels of 5.6, 6.0, 6.7, 7.0, 7.5 and 8.2, respectively. The percentages of zoospore infections were 8.0, 26.67, 66.67, 50.67, 61.33 and 48.0% for pH levels of 5.6, 6.0, 6.7, 7.0, 7.5 and 8.2,

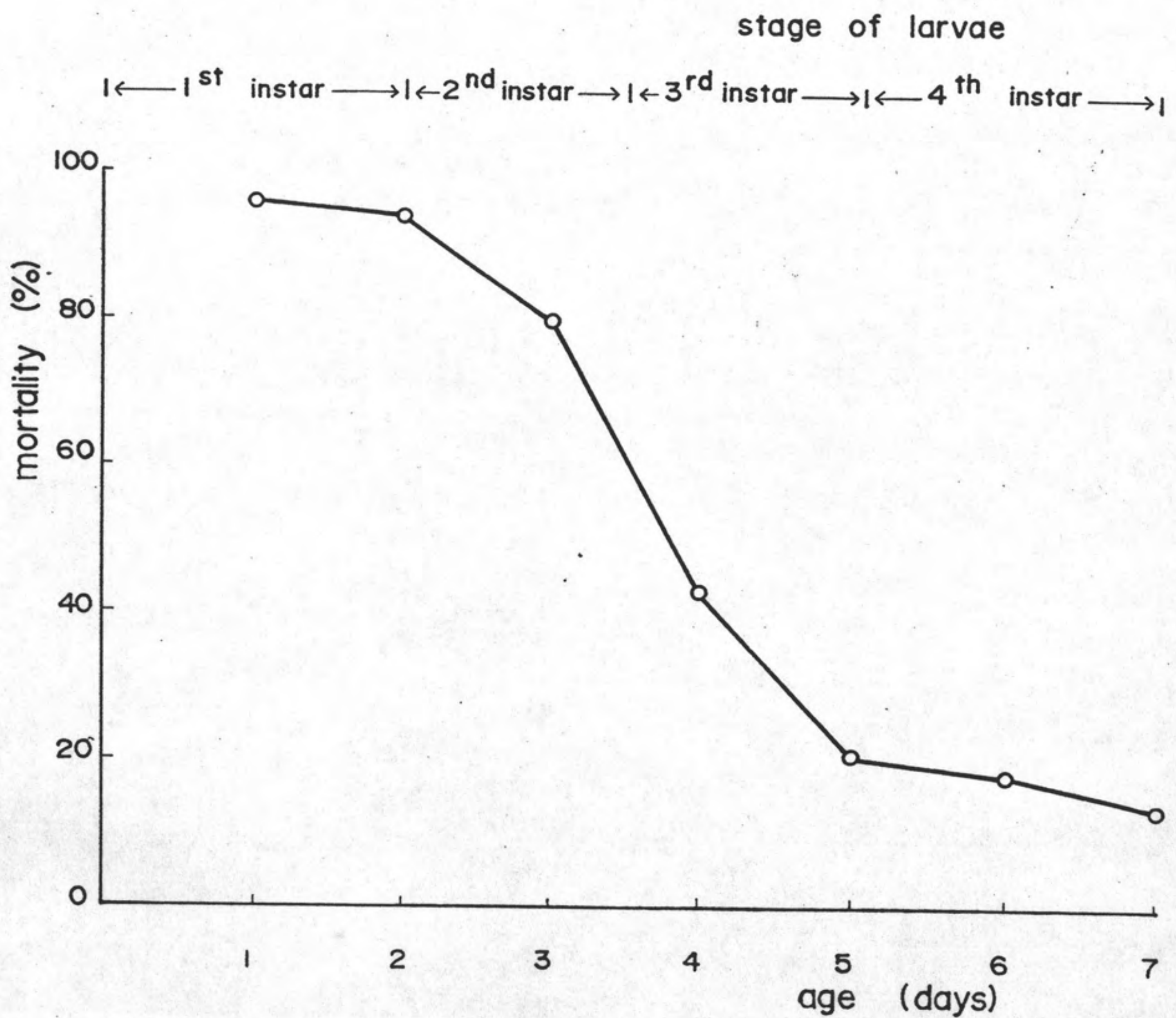

Fig. 24 Percentage of mortality of Ae. aegypti at various ages following inoculation of L. giganteum

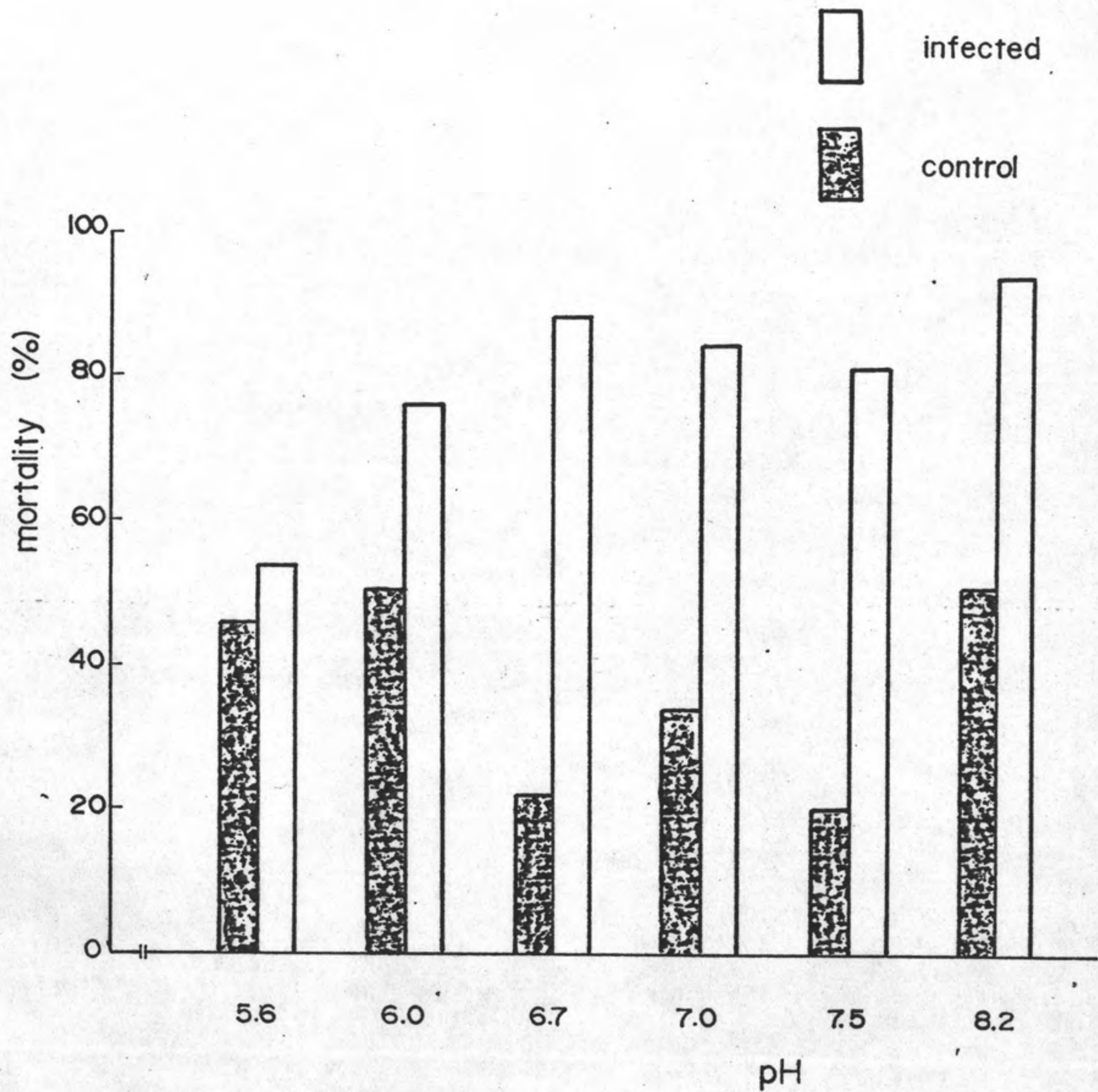

Fig. 25 Percentage of mortality of 2-day-old, Ae. aegypti larvae following treatment with L. giganteum in different pHs of water

respectively. The results showed that in the water which had pH levels at 6.7 was better zoospore infection.

Effects of temperature on the infection

The results of various temperatures on mortality rates of *Ae. aegypti* larvae by *L. giganteum* are shown in Fig. 26. Data showed that the percentages of infection of *Ae. aegypti* were 0, 57.33, 62.0, 48.0, 32.9 and 0% for the temperatures of water at 12, 20, 25, 28, 30 and 27 ^{o}C. From Fig. 26 the optimal temperatures for infections were between 20 and 30 ^{o}C, but the highest infection rate occurred at 25 ^{o}C, 62.0% infection. There was a marked decrease in the infection rate above 28 ^{o}C and below 20 ^{o}C. At the temperatures of 12 ^{o}C and 37 ^{o}C, all the mosquito larvae in the inoculated and control beakers died.

Effect of various types of water to the infection by *L. giganteum*

Five different types of water from different places, i.e., Klong Mahanak, slum at Klong Toey, high tide and low tide water from Chao Praya river at Sathupradid, water from the pond near Chulalongkorn University Hall, pond water with aeration by fountain from the pond in front of Chulalongkorn University and rain water from reservoir.

The results in Fig. 27 show the amounts of BOD, COD and acidity of the sampled water. BODs of the various waters were 60.3 mg/l for Klong Mahanak, 28.215 mg/l for low tide water, 12.3 mg/l for slum, 9.786 mg/l for pond water, 6.04 mg/l for pond water with aeration, 3.63 mg/l for high tide water and 2.407 mg/l for rain water. CODs were 200.80 mg/l for Klong Mahanak, 84.34 mg/l for slum, 74.0 mg/l

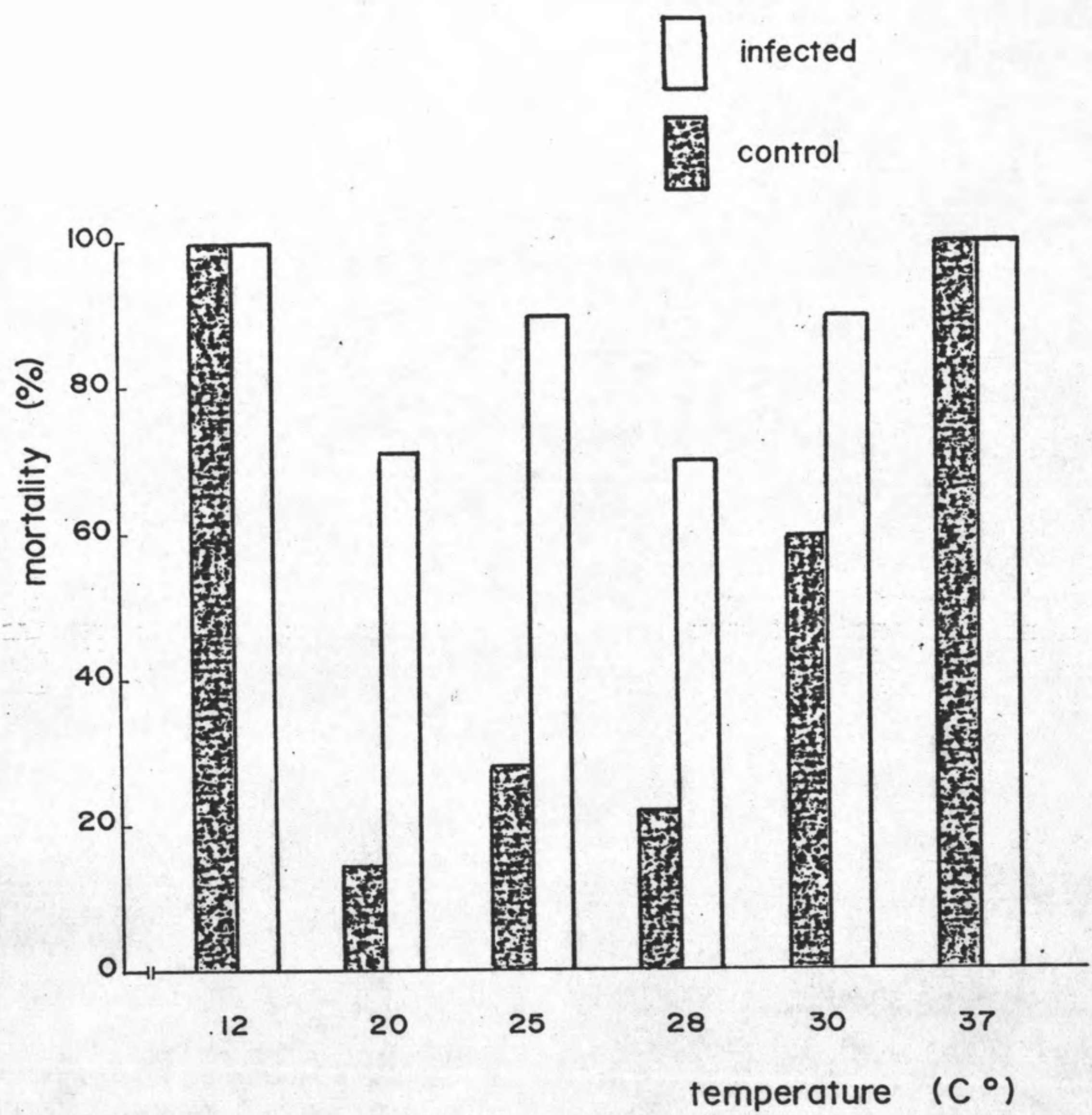

Fig. 26 Percentage of mortality of 2-day-old Ae. aegypti infected by 6.0×10^6 zoospores per ml. of L. giganteum cultured on SFE agar for 5 days at various temperatures.

BOD
COD
pH

pH COD BOD

10 200 100
8 80
6 60
100
4 40
2 20
0 0 0

klong mahanak
low tide
slum
pond water
fountain pond water
high tide
rain water

kinds of habitat

Fig. 27 The pH, BOD, COD of various types of water which were used for infection experiments.

for low tide water, 50.6 mg/l for pond water, 19.920 mg/l for pond water with aeration, 16.0 mg/l for high tide water and 12.936 mg/l for rain water. The acidities of the various waters were 8.42 for Klong Mahanak, 8.11 for slum, 7.35 for low tide water, 6.82 for high tide water, 6.62 for rain water, 6.42 for pond water and 6.06 for pond water with aeration. From these results, the two types of water that had the lowest BOD and COD were high tide water and rain water. The acidities of these two types of water were also at the optimum pH for larval infection (Fig. 25). Water from high tide water and rain water should be good for use as a medium or habitat for the infection of L. giganteum in Ae. aegypti.

Effect of BOD and COD on infection

Investigation on the effects of BOD and COD on zoospore infection was done. Zoospores were produced by the method described earlier in 4; the culture of L. giganteum grown on SFE agar for 5 days and then blended with distilled water. The inoculum size was 23.4 cm^2 result from Fig. 23. The different types of BOD and COD used for this study were from various waters from different places where BOD, COD and pH were measured before use, (Fig. 27), i.e., water from Klong Mahanak, Slum at Klong Toey, high tide and low tide water of Chao Praya river at Sathupradid, and rain water, pond water and pond water with aeration. The data are presented in Fig. 28, 29.

The results in Fig. 28, 29 showed the percentage of mortality of 2-day-old Ae. aegypti larvae with 23.4 cm^2 area of inoculum size. After 72 hours, the percentage of dead larvae in each type of BOD and COD were 59.0, 47.0, 90.0, 65.0, 57.33, 84.5 and 76.5% (experimental

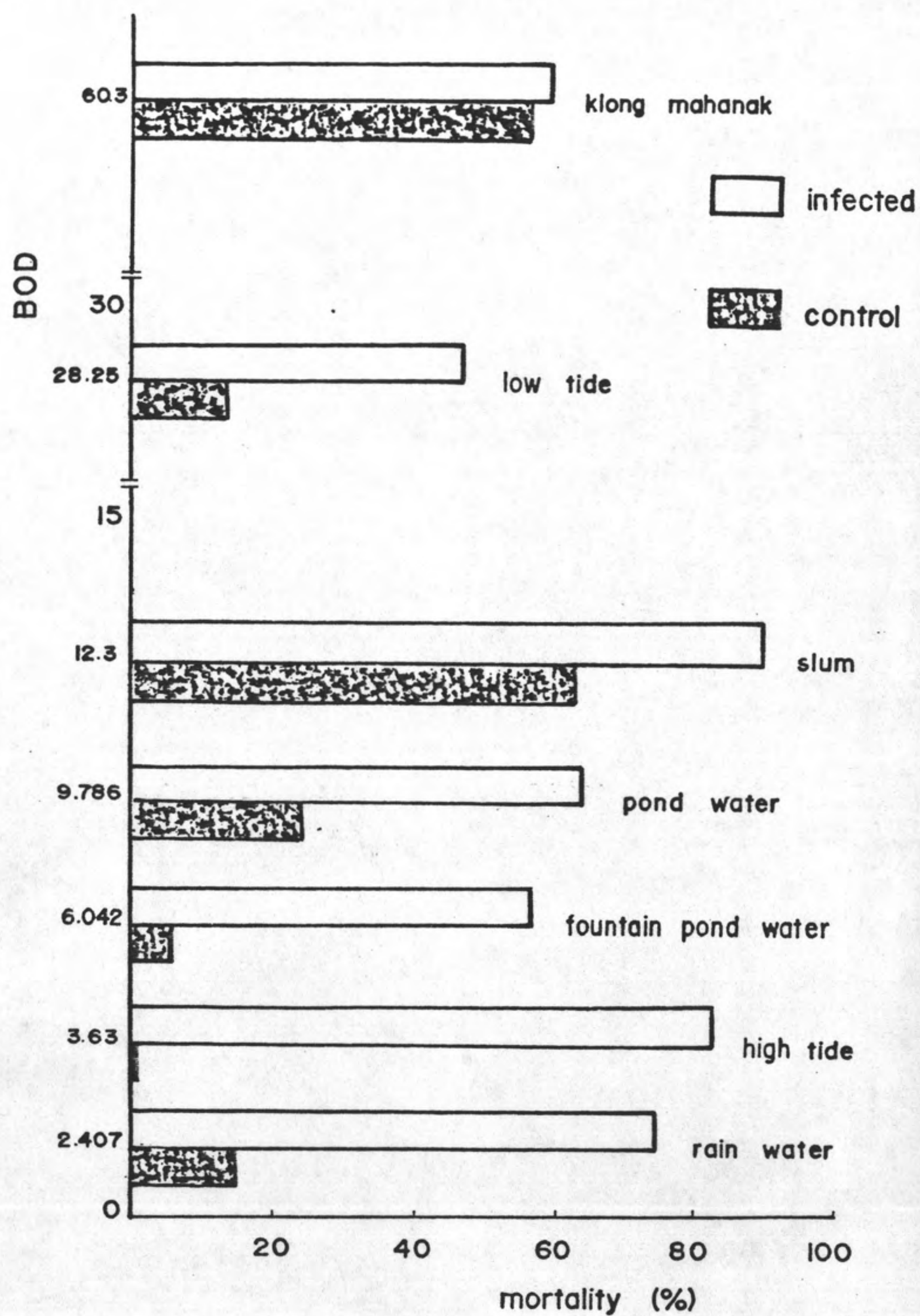

Fig. 28 Percentage of mortality of _Ae_. _aegypti_ after infection by _L_. _giganteum_ in various types of BOD

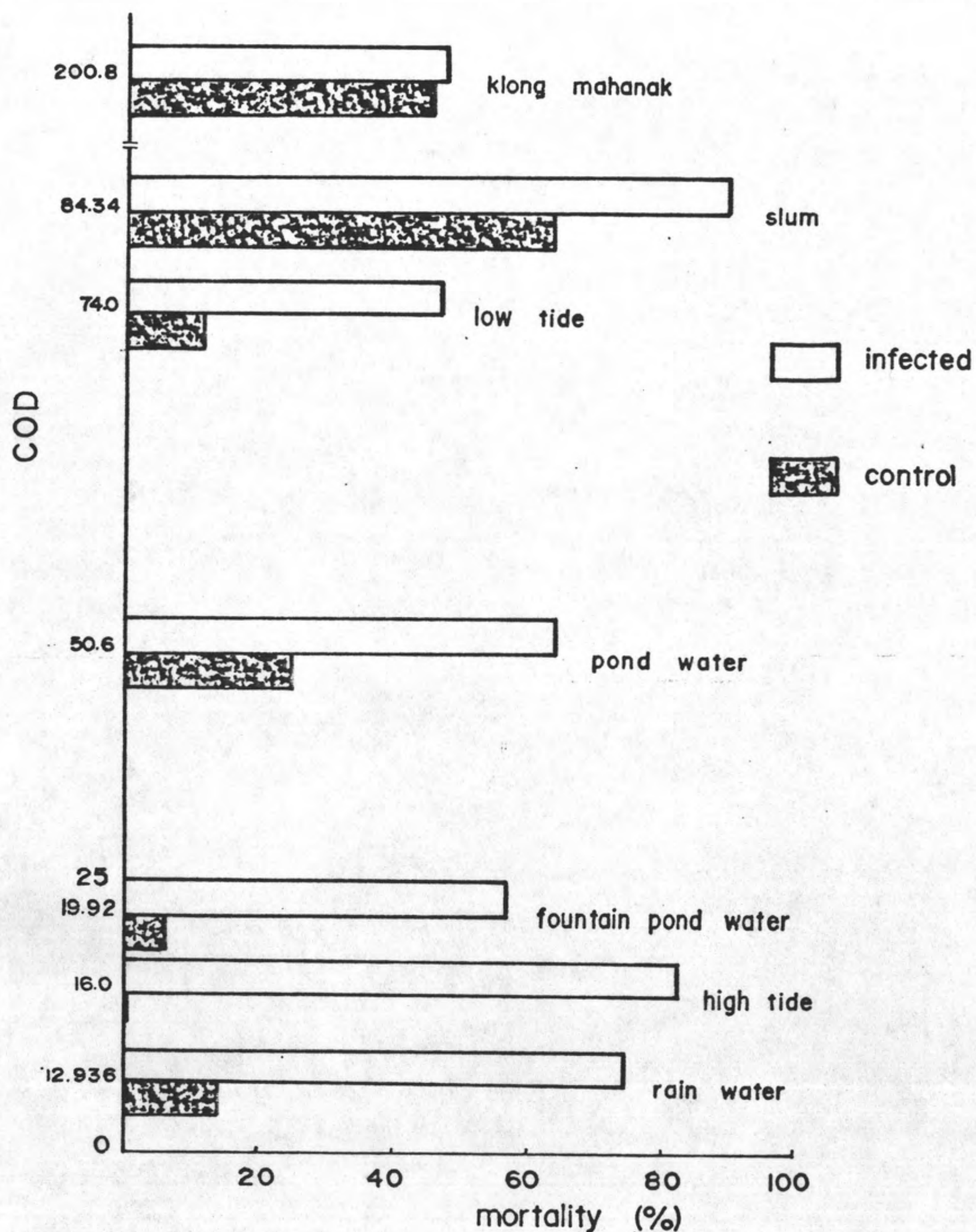

Fig. 29 Percentage of mortality of Ae. aegypti after infection by L. giganteum in various types of COD

beakers) and 56.0, 12.0, 64.0, 15.0, 6.0, 0.0 and 16.0% (control beakers) in Klong Mahanak, low tide, slum, pond water, pond water with aeration, high tide and rain water, respectively. The data were separated based upon BOD and COD for Fig. 28 and Fig. 29. From these results, the highest percentages of infection occurred in high tide water and rain water, supported by the results from Fig. 27.