INVESTIGATION OF MOSQUITO LARVAE KILLING FUNGI FROM MOSQUITO LARVAE IN THE NORTHERN THAILAND

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ABSTRACT

Mosquitoes cause annoyance and are vectors of many medically important diseases. Thus, mosquito controls are needed to reduce numbers of mosquitoes. At present, we begin to pay attention to the biological control because we faced the problem that mosquitoes have developed resistance to chemical agents. This research project was the preliminary study on the isolation of fungi from mosquito larvae collected from aquatic habitats in the northern Thailand, the larvicidal activity of these fungal isolates in laboratory and their growth characteristics. Mosquito larvae samples were collected from 100 aquatic habitats. Thirty two isolates of fungi were isolated from 49 infected cadavers. After the larvicidal activity against laboratory colonies was tested, only 10 isolates exhibited more than 50% mortality. Three isolates were selected according to their high killing ability and obvious sign of fungal infection. The isolate No. 12A-6 appeared to be *Trichoderma viride* had LC_{50} values against *Culex quinquefasciatus, Aedes aegypti, Anopheles dirus,* and *Anopheles minimus* at concentration of 7.16×10^5, 1.29×10^6, 1.40×10^6, and 1.71×10^6 spores/ml, respectively. The optimal conditions for growth was at 20-25 $^{\circ}$C and at pH 5-6. This fungus could assimilate all the carbon and nitrogen sources tested. The isolate No. 10A-15W which was identified as *Beauveria sp.* demonstrated larvicidal activity against all 4 species of mosquito larvae with LC_{50} values at 2.43×10^6,

2.92 x 10^6, 1.18 x 10^6, and 3.96 x 10^6 spores/ml, respectively. The optimal conditions for growth of this fungus was at 20-25 °C and at pH ranges 6-8. This fungal isolate could assimilate all the carbon and nitrogen sources tested. The isolate No. 29B-5W which was identified as *Aspergillus niveus* could kill those 4 species of mosquito larvae with LC_{50} values at 1.79 x 10^4, 1.59 x 10^5, 6.73 x 10^4, and 3.86 x 10^5 spores/ml, respectively. It was believed that the toxin of these two fungal isolates (No. 10A-15W & 29B-5W) might contribute to this larvicidal activity. The optimal conditions for growth of isolate No. 29B-5W was at 25-37°C and at pH range of 5-8. This fungus could also assimilate all the carbon and nitrogen sources tested. Moreover, there were two interesting isolates of fungi which, one was isolate No. 35C-1 which was identified as *Aphanomyces sp.* and the another one was isolate No. 36A-LK which was identified as *Leptolegnia sp.* Both isolates were aquatic fungi which infected mosquito larvae and obviously showed sign of fungal infection in mosquito larvae. The isolate No. 35C-1, *Aphanomyces sp.*, grew well at 20-37 °C and at pH 5-8. Peptone and glucose were used as the sole sources of nitrogen and carbon for growth. Whereas the isolate No. 36A-LK, *Leptolegnia sp.*, grew well at 20-25 °C and at pH 6-8. Peptone was used as the sole source of nitrogen; glucose and maltose were equally excellent as carbon sources.

บทคัดย่อ

ยุงนอกจากจะเป็นสาเหตุทำให้เกิดความรำคาญแล้วยังเป็นพาหะนำโรคที่สำคัญทางการแพทย์หลายโรค ดังนั้นวิธีควบคุมและกำจัดยุงจึงมีความจำเป็นต้องใช้เพื่อลดจำนวนยุง ในปัจจุบันนี้เราเริ่มหันมาสนใจชีววิธีในการควบคุมและกำจัดยุง เนื่องจากพบปัญหาว่ายุงเริ่มดื้อต่อสารเคมี ดังนั้นการค้นคว้าวิจัยเรื่องนี้จึงเป็นการศึกษาเบื้องต้นเกี่ยวกับการแยกเชื้อราจากลูกน้ำ ซึ่งเก็บจากแหล่งน้ำในเขตภาคเหนือของประเทศไทย ความสามารถในการฆ่าลูกน้ำของเชื้อราที่แยกได้ในห้องปฏิบัติการ และศึกษาคุณลักษณะการเจริญของเชื้อราเหล่านี้ จากตัวอย่างลูกน้ำซึ่งเก็บจากแหล่งน้ำ 100 แหล่ง มีเชื้อรา 32 สายพันธุ์ที่สามารถแยกได้จากลูกน้ำที่ติดเชื้อราจำนวน 49 ตัว หลังจากทดสอบความสามารถในการฆ่าลูกน้ำในห้องปฏิบัติการแล้ว พบว่ามีเพียง 10 สายพันธุ์ที่มีคุณสมบัติในการฆ่าลูกน้ำได้ และ 3 สายพันธุ์ถูกคัดเลือกเนื่องจากมีความสามารถในการฆ่าลูกน้ำได้สูง และพบลักษณะการติดเชื้อราได้ชัดเจน เชื้อราสายพันธุ์ที่ 12A-6 ซึ่งเป็นชนิด *Trichoderma viride* สามารถฆ่าลูกน้ำยุงชนิด *Culex quinquefasciatus, Aedes aegypti, Anopheles dirus* และ *Anopheles minimus* และมีค่า LC_{50} ที่ความเข้มข้น 7.16×10^5, 1.29×10^6, 1.40×10^6 และ 1.71×10^6 สปอร์/มล. ตามลำดับ สภาวะที่เหมาะสมในการเจริญของเชื้อคือ ที่อุณหภูมิ 20-25 °ซ และที่ pH 5-6 เชื้อรานี้สามารถใช้คาร์บอนและไนโตรเจนจากแหล่งคาร์บอนและไนโตรเจนที่ใช้ในการทดสอบได้ทุกชนิด เชื้อราสายพันธุ์ที่ 10A-15W ซึ่งเป็นชนิด *Beauveria* sp. มีคุณสมบัติในการฆ่าลูกน้ำทั้ง 4 ชนิดได้และมีค่า LC_{50} ที่ความเข้มข้น 2.43×10^6, 2.92×10^6, 1.18×10^6 และ 3.96×10^6 สปอร์/มล. ตามลำดับ สารพิษของเชื้อราชนิดนี้มีผลในการฆ่าลูกน้ำด้วย สภาวะที่เหมาะสมในการเจริญของเชื้อนี้คือ ที่อุณหภูมิระหว่าง 20-25°ซ. และที่ pH 6-8 เชื้อนี้สามารถใช้คาร์บอนและไนโตรเจนที่ใช้ในการทดสอบได้ทุกชนิด เชื้อราสายพันธุ์ที่ 29B-5W ซึ่งเป็นชนิด *Aspergillus niveus* สามารถฆ่าลูกน้ำทั้ง 4 ชนิดได้และมีค่า LC_{50} ที่ความเข้มข้น 1.79×10^4, 1.59×10^5, $6.73 \times$

10^4, และ 3.86×10^5 สปอร์/มล. ตามลำดับสารพิษของเชื้อรานี้สามารถฆ่าลูกน้ำได้เช่นกัน สภาวะที่เหมาะสมในการเจริญของเชื้อราชนิดนี้คือ ที่อุณหภูมิ 25-37 °ซ และที่ pH 5-8 เชื้อนี้สามารถใช้คาร์บอนและไนโตรเจนที่ใช้ในการทดสอบได้ทุกชนิด นอกจากนี้ยังพบเชื้อราที่น่าสนใจอีก 2 สายพันธุ์ คือ ชนิด *Aphanomyces sp.* สายพันธุ์ที่ 35C-1 และชนิด *Leptolegnia sp.* สายพันธุ์ที่ 36A-LK ซึ่งทั้ง 2 ชนิดนี้เป็นราน้ำซึ่งก่อให้เกิดโรคในลูกน้ำ และแสดงลักษณะการติดเชื้อราในลูกน้ำได้ชัดเจน *Aphanomyces sp.* สายพันธุ์ที่ 35C-1 เจริญได้ดีที่อุณหภูมิ 20-37°ซ. และที่ pH 5-8 สามารถใช้ไนโตรเจนจากเปปโตนเท่านั้นและใช้คาร์บอนจากน้ำตาลกลูโคส ขณะที่ชนิด *Leptolegnia sp.* สายพันธุ์ที่ 36A-LK เจริญได้ดีที่อุณหภูมิ 20-25 °ซ. และที่ pH 6-8 เชื้อราชนิดนี้สามารถใช้ไนโตรเจนจากเปปโตนเท่านั้นและคาร์บอนจากน้ำตาลกลูโคสและมอลโตส

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I. INTRODUCTION

Mosquitoes are one of the major insect problems of the world and are the most prominent of the blood-sucking arthropods that annoy man and other warm-blooded animals. In Thailand, they are vectors of pathogens that cause malaria, dengue haemorrhagic fever, and filariasis. Thus, mosquito controls are needed to reduce their annoyance and the transmission of disease to man or domestic animals. For the beginning of the mosquito control, the programs relied principally on chemical control, consisting of DDT, organophosphate compounds and others. They were very effective broad-spectrum insecticides. Later we faced problems such as the mosquitoes had developed resistance to DDT and to all of the associated hydrocarbon insecticides (World Health Organization, 1980b; Brown, 1986) and DDT provided a prolonged residual effect, maintained its toxic properties in the environment (World Health Organization, 1970, 1971). Whereas the occurrence of mosquito-borne diseases had been increasing, insecticide resistance and higher costs of newer insecticides were major factors contributing to the problem and, along with environmental considerations, attention on the need for alternatives or supplements to conventional chemical insecticides for mosquito control had been focused (Pont *et al.*, 1977).

To date, the alternative measure for mosquito control are biological control and the effective method in reducing the mosquito

population is to eradicate at larval stages because they prefer to accummulate in aquatic habitats which are more convenient to be controled. For these reasons, increasing attention has been directed toward natural enemies such as predators, parasite and pathogens of mosquito larvae. The natural enemies of mosquitoes in aquatic habitat of which fish are the most commonly used, the best known is *Gambusia affinis* (Gall *et al.*, 1980; Bheema Rao *et al.*, 1982). This kind of fish can tolerate relatively high salinities, though more sensitive to brackish water, can withstand higher organic pollution and so is better suited for controlling *Culex quinquefasciatus.* This fish is useful against mosquitoes breeding in temporary standing water or man-made breeding sites but it can't survive in temporary habitats that periodically dry out and in running water, this severely limits its usefulness (Wongsiri and Andre, 1984). Of the invertebrate predators considered for biological control, *Toxorhychites* mosquitoes can be contemplated for control of mosquitoes breeding in container habitats. Disadvantages of this species are found later that they have to repletedly reintroduce to mosquito larval habitat (Gerberg *et al.*, 1978) and laboratory rareing of *Toxorhynchites* gave low yield of larvae and pupae (Focks and Boston, 1979; Annis and Rusmiarto, 1988). Nematodes are among the more common contenders for mosquito control, the best known is *Romanomermis culicivorax* which infects at least 16 mosquito species naturally and over 80 species can be experimentally infected (cited in Service, 1983). However, *Romanomermis culicivorax*

cannot tolerate even slightly saline water, polluted waters and low oxygen concentrations (cited in Service, 1983). Elevated calcium, nitrates, nitrites and phosphates inhibited infection of *R.culicivorax* (Brown and Platzer, 1978). Aquatic fauna including beetles, dragonfly nymphs, and copepod appear to be predators of the pre-and post-parasitic stages (Platzer *et al.*, 1980; cited in Service, 1983). The most promising biological control agents for mosquitoes are *Bacillus thuringiensis israelensis (Bti)* and *Bacillus sphaericus* 1593. They are microbial insecticides acting as a stomach poison but these species do not persist in the environment. Consequently they have to be repeatedly applied. Moreover, suspended particulate matter, organic and inorganic pollutions reduce its efficiency, and in deep water it tends to settle down amongst bottom sediments so that little is ingested by mosquito larvae. *Bti* is more effective against *Aedes* and *Culex* than *Anopheles* larvae. Most *Culex* species, including *Cx.quinquefasciatus*, are highly susceptible to *B. sphaericus*. In general, *Anopheles* are more susceptible to *B. sphaericus* than they are to *Bti*. Particulate matter, strong ultraviolet radiation, low temperatures and alkalinity decrease larvicidal activity and as with *Bti* rapid settleing of toxic entities result is reduced larval mortality. But *B. sphaericus* has good potential and the ability to recycle. Thus, it is ecologically more interesting than *Bti* (Cited in Service, 1983; Silapanuntakul *et al.*, 1983; Wongsiri and Andre, 1984; McGaughey , 1985). It is clear that a large reservoir of potentially

along the way will certainly discover other perhaps even more useful candidates. Fungi are introduced to be evaluated as potential mosquito larvicides. Many fungi have mosquito infecting capacity such as *Coelomomyces, Lagenidium, Leptolegnia, Culicinomyces, Metarhizium,* and *Tolypocladium,* etc. (Robert and Castillo, 1980; Davidson and Sweeney, 1983; Miller *et al.*, 1983). For mosquito larvae killing fungi, one immediately thinks of *Coelomomyces.* Fungi of this genus are obligate parasites most commonly reported from mosquito larvae. Hembree (1979) reported that *Coelomomyces sp.* infected mosquitoes in the field and laboratory. Although they are not particularly promising candidates as a potential biological agent at present because they have a complicated and incompletely understood life cycle involving an obligatory sexual cycle in copepods before they can infect mosquitoes. Furthermore, cultures cannot be established on artificial media (Couch, 1972; Whisler *et al.*, 1975; World Health Organization, 1980). Nevertheless, as highly specific obligate parasites which seek out mosquito larvae, they may prove extremely valuable for vector suppression in the future. Other fungi can be relatively easily cultured on artificial media, and commercial preparations. Among the better known fungus is *Culicinomyces clavosporus* which produces mortality in larvae of several major mosquito genera but its problems are that, large volumes are necessary because of low spore production in cultures and it is intolerant to high salinities and temperatures above 30°C (Couch *et al.*, 1974; Knight, 1980). *Lagenidium giganteum*

is another fungus which can grow in artificial media (Jaronski and Axtell, 1983, 1984; Guzman and Axtell, 1986). It has caused relatively high mortalities in some trials, especially against *Culex* and *Aedes* mosquitoes. Recycling can be quite good and *Lagenidium* can withstand drying out. It was believed to have control potential (Jaronski and Axtell, 1982; Guzman and Axtell, 1987; Kerwin and Washino, 1987). There are other fungi which have been presently studied and have a potential to be further developed to biological control agents. Some fungi can produce toxin such as *Beauveria bassiana* and *Metarhizium anisopliae* (Suzuki *et al., 1977;* Deacon, 1984). Moreover, there is aquatic fungi *Leptolegnia sp.* which can produce infective zoospores in aquatic habitats where mosquito larvae lived. (Seymour, 1984; Nnakumusana, 1986). There has been much interest in use of fungi as control agents of mosquitoes. Many potential control agents have been identified (Roberts and Strand, 1977; Roberts and Castillo, 1980) and it revealed that relatively few of the known mosquito pathogens had been reported from developing nations with serious mosquito-borne disease problems especially Thailand and that almost none of these had been systematically evaluated as potential biological control agents. However, most of these represent limited collections and very little is known about their epizootiology. This reflects a need for concentrated research effort on the mosquito larvae killing fungi as potential biological control agents of mosquitoes and in general, the use of indigenous rather than introduced new pathogens should be

encouraged because introduced pathogens may not survive their new ecosystem (World Health Organization, 1982). Therefore, the present study is made to survey and isolate mosquito larvae killing fungi from mosquito larvae collected from 100 aquatic habitats in northern Thailand provinces and also to provide additional knowledge of the host range of such agents. However, for a potential biological control agent, the successful demonstration of its pathogenicity towards mosquito larvae is not alone sufficient unless the agent is also amenable to mass production so the optimal condition for growth of mosquito larvae killing fungi are also studied. Thus, the main objectives of this study are as follows:

1. Isolation of fungi from mosquito larvae collected from aquatic habitats in the northern Thailand.

2. Study of ability of isolated fungi in killing mosquito larvae in laboratory.

3. Cultivation of mosquito larvae killing fungi in various media and studies of the optimal conditions for growth of those fungi in laboratory.

II. LITERATURE REVIEW

Mosquitoes (Culicidae: Diptera) enjoyed the reputation of being among those pests of man and animals which were most persistent, irritating and difficult to control. They were responsible for more human illnesses than any other group of arthropods in the tropics and subtropics.

Mosquitoes as disease vectors were most severe in Thailand such as *Culex quinquefasciatus*, the house mosquito, bred prolifically in large areas of Thailand, in organically polluted waters of all types, including lagoons for animal waste disposal, and it was often an annoying biter and disease vector such as bancroftian filariasis (Rao *et al.*, 1981; Sucharit, 1988).

Dengue was one of the most important disease, caused by dengue virus, a member of Flavivirus and belonged to the arbovirus group which was transmitted through the bite of mosquitoes *Aedes aegypti*. Ever since dengue haemorrhagic fever (DHF) was first recognized as an epidemic disease of children in Bangkok in 1958 it has become one of the major public health problems in Thailand. There were only 2,148 cases with 240 deaths reported from Bangkok city. Then the incidence increased to 5,947 cases in 1962 when the disease started to spread to other big cities, to which the communications with Bangkok were accessible. In the next ten years dengue has spread throughout the country with the total number of 23, 769 and 38, 768

cases in the years 1972 and 1977 respectively. With big outbreaks in the North and Northeastern regions of Thailand in 1984 - 1985, the incidence has reached its highest peak ever for the period of 28 years. There were 69,101 cases with 496 deaths in 1984 and 80,811 cases with 505 deaths in 1985 (Ministry of Public Health, Annual report, 1984, 1985; cited in Suchitra, 1987). The number of DHF reported cases has increased by 28.6 folds from 1958 to 1985 (Suchitra, 1987). Cases of dengue from all the 73 provinces in 1988 were reported to the Division of Epidemiology (Ungchusak, 1989). The incidence of dengue haemorrhagic fever, dengue shock syndrome and dengue fever was 45 per 100,000 population and the case-fatality rate was 0.56 percent and the north - eastern region had the highest incidence.

However, one of the most important disease in Thailand which had *Anopheles minimus* and *Anopheles dirus* as main vectors was malaria. Eventhough the incidence of malaria has been greatly reduced in the past 45 years but it remained as a public health problem in Thailand as if mosquitoes had not been eliminated. The mortality rate in 1943 was 351 with a death rate of 300 per 100,000 population. During 1966-1972 malaria morbidity decreased to 2.2-3.6 per 1,000 population. Thereafter, there was an annual rise in malaria cases. The morbidity rate in 1978 was 7.1 and reached 10.6 per 1,000 population in 1981. This increase was mainly attributable to population migration and the emergence of drug resistance of malaria parasites. However, a decline

in malaria cases was seen in 1982 with the Annual Parasite Incidence (API) of 9.1 per 1,000 population. The APIs during 1983-1987 were 5.0-5.9 per 1,000 population. Currently, area with a very high incidence of malaria are some provinces along the Thai-Kampuchean and Burmese borders and some southern provinces. In 1987, the highest incidence of malaria was reported in Trad, a Thai-Kampuchean border province, with an API of 97.5 per 1,000 population. Tak, a province near the Burmese border, faced the same problem with an API of 78.0 per 1,000 population (Malikul, 1989). Thus, mosquito controls are needed to reduce their annoyance and the transmission of disease to man or domestic animals.

Mosquito control

The primary purpose of mosquito control was to eliminate mosquito-borne disease which caused death and debility. From the beginning of the first control, the method relied principally on chemical control, consisting of routine oil and pesticide spraying of mosquito-breeding habitats, and environment management (Thevasagayam, 1985; Rojanapremsuk *et al.*, 1986). Later biological control was introduced to reduce mosquito population.

A. Chemical control

The discovery in the early 1940s, that DDT was a very effective insecticide for control of insect of public health importance, prompted the world-wide to embark on a mosquito eradication programme. At the beginning of mosquito control, the programs relied principally on chemical control, consisting of DDT, organophosphate compounds and others because they were very effective broad-spectrum insecticides. Later the problem of vector resistance and in some cases malaria control program mismanagement caused difficulty in eradication. Resistance to DDT by mosquito vectors was now very widespread (World Health Organization, 1980; Rawling *et al.*, 1985; Brown, 1986) and only a few countries can afford the more expensive alternatives such as the organophosphates, synthetic pyrethroids and carbamates. But now, in the fifth report (World Health Organization, 1980) the WHO Expert Committee on Vector Biology and Control listed 51 anopheline species, 42 culicines and 41 other arthropods of public health or veterinary importance as resistant to one or more insecticides eventhough organophosphorus insecticides (Takahashi and Yasutomi, 1987; Yasutomi and Takahashi, 1987). The use of chemical insecticide were possible hazards to man arising from the occurrence of residues of insecticide in foods (World Health Organization, 1971) and in 1986 Levine reported that the number of deaths globally from pesticide poisoning has been estimated at about

80,000 per annum, using a case fatality rate of 0.5% for the developing countries and one of 0.25% for the developed countries (cited in Goulding, 1988). In many parts of the world, studies of residual levels of these toxic substances in the environment, foods, directly exposed people, and indirectly exposed people have indicated levels of contamination that have encouraged restrictions on the manufacture and use of these products. In accordance with the recommendations made in 1969 (World Health Organization, 1970). Several countries have stopped using DDT except in situations where no satisfactory substitute was available. Current concern over the potential hazard from DDT was based on (1) its ubiquity, (2) its persistence in the environment and the effect on some wildlife, (3) its retention in living organisms, (4) its capacity to be transferred to and to be retained in the foetus, and (5) the existence of some experimental evidence of its capacity to induce tumours in experimental animals. Although the available experimental data did not provide sufficient information to allow a definite evaluation of the potential carcinogenicity of DDT, they did strongly indicate that it could not be ignored.

Because of the emergence and spread of insecticide resistance in many species of mosquitoes, the high cost of new types of chemical insecticides and concern with environmental pollution, made it apparent that mosquito control could no longer be solely dependent on the use of chemicals. Alternative methods of control had to be developed.

B. Biological control

1. Biological pathogens of mosquitoes.

Larvivorous fishes

Fish is one of the most important natural regulatory agents of mosquito. One hundred and eighty nine species of fish around the world were predators of mosquito larvae (Jenkin, 1964). The best known mosquito fish, *Gambusia affinis,* was a samll species which had become widely distributed throughout the world since its use in mosquito control programs began in about 1905. It adapted to a wide range of environments and was found in fresh water, brackish water, and salt marshes with salinity concentrations above those of sea water. The mosquito fish also could withstand a wide range in temperature and very low oxygen levels in the water, since it had evolved the behavior of skimming the surface of the water where the levels were highest because of oxygen exchange from the air. Mosquito fish fed on aquatic insects and insect larvae. The females were fertilized internally, and the young were born alive after a gestation period of 15 to 50 days, depending on water temperature. The female could retain sperm for an extended period of time and produced several broods without mating again. Fertility and number of young fish were increased with increasing feed intake and size of the female, but

fertility was decreased as the female became older (Gall *et al.*, 1980). Mosquito fish such as *Poecilia reticulata* and *Oreochromis niloticus* were introduced into Thailand and the result of using *O. niloticus* as a biological control agent of mosquito larvae in laboratory showed that they could eat *Culex sp.* at the rate of 321±136 larvae per day for one fish (size 1.1-2.0 cm. long), 349 ± 189 larvae per day for one fish (size 2.1 - 3.0 cm. long) and 476 ± 163 larvae per day for one fish (size 4.1 - 5.0 cm. long) (cited in Wongsiri and Andre, 1984). Such differences reflected the level of genetic control and indicated that selection for large size would be an effective breeding method. It also could be assumed that larger, faster-growing fish would eat more insect larvae. The mosquito fish, *Gambusia affinis,* had been shown to be quite effective at reducing mosquito populations in field tests in some experiments (Hoy and Reed, 1971; Hoy *et al.*, 1971; Bheema Rao *et al.*, 1982) but quite ineffective in other experiments (Hoy *et al.*, 1972; Cech and Linden, 1987; Kramer *et al.*, 1987; Kramer *et al.*, 1987). Assessments of green sunfish, *Lepomis cyanellus,* as regulators of mosquito populations in rice fields had also yielded inconsistent results (Davey and Meisch, 1977). For a biological control agent to be effective at reducing a pest population, it must have high spatial overlap with the pest. Part of the inconsistency of larvivorous fishes to control mosquitoes in rice fields might be due to differences in overlap between predator and prey in different fields. Factors such as water depth and type and

density of vegetation vary considerably among rice fields and could cause overlap between mosquitoes and fishes to vary. However, use of larvivorous fish such as *Gambusia* would appear to be the cheapest method and the use of indigenous rather than introduced fish should be encouraged because indigenous fish were better adapted to local conditions and also were established easily and economically with community help in rearing (World Health Organization, 1982) but the limiting factors in the use of larvivorous fish were the mortality of fish resulting from pits drying up during the hot months and they had to frequently apply in habitats and presence of floating debris and algae could prevented effective predation (Bheema Rao *et al.*,1982).

Romanomermis culicivorax

Romanomermis culicivorax is an obligatory endoparasitic nematode, the larvae of which completed their development inside mosquito larvae. It was relatively easy to mass produce (Petersen and Willis, 1972; Petersen *et al.*,1978). Reviewed by Service, there was a commercial product known as "Skeeter Doom" but eggs exhibited reduced viability following transportation. Another company expressed interest in culturing *R. culicivorax* but lost it when a feasibility study indicated that there would be poor financial returns on developing biocontrol agents. *Romanomermis culicivorax* infects at least 16 mosquito species naturally and over 80 species could be

experimentally infected (cited in Service, 1983); *Anopheles* seems to be the most susceptible. Water depth did not appear important because preparasites concentrated near the surface and thus had a high degree of contact with mosquito larvae. In El Salvador, however, wave action along Lake Apastepeque reduced its effectiveness against *Anopheles albimanus* (Petersen *et al.*, 1978). Moreover, a mean infection rate of 96% dropped to 74 and 89% when applications were made before heavy rain (Willis *et al.*, 1980). It seems that although *R. culicivorax* may have a role in biological control of mosquitoes, effective long term control is not likely to occur from a few artificially created epizootics (Zaim *et al.*, 1988). Further, the technical procedures of production, storage and transportation of the nematode make it costly to use it for periodic inundative releases for immediate control. Thus, *R. culicivorax* seems to be of limited use. Moreover, *Romanomermis culicivorax* could not tolerate even slightly saline water, polluted waters and low oxygen concentration (cited in Service, 1983). Elevated calcium, nitrates, nitrites, and phosphates inhibited infection (Brown and Platzer, 1978). Aquatic fauna including bettles, dragonfly nymphs and copepods appeared to be predators of the pre - and post parasitic stages (Platzer and MacKenzie - Graham, 1980; cited in Service, 1983).

Toxorhynchites

Toxorhynchites is a genus of large mosquito which is not hematophagous as an adult, and its larval instars are predators of small invertebrates, including larvae of other mosquitoes. Several of the more important mosquito vectors of human disease breed in discarded cans, bottles, water cisterns, and tree holes. Control of larvae of these species is difficult because the larval habitats are small, dispersed and often inaccessible. Mosquitoes of the genus *Toxorhynchites* are larval predators of container-breeding mosquitoes. It has been demonstrated that in some situations the genus *Toxorhychites* has potential as a biological control agent against container-breeding mosquitoes (Gerberg and Visser, 1978). In this connection, inundative release of adult *Toxorhynchites* was proposed as necessary to upset the normal predator-prey relationship but inundative releases implied the mass rearing of large numbers of mosquitoes and in determining the practical utility of *Toxorhynchites* as a biological control agent, the cost of mass rearing could be as important a parameter as the biological aspects of the mosquito. Such as one *Tx. rutilus rutilus* larvae required ca. 100 *Aedes aegypti* as food (Focks *et al.*, 1977); thus a rather large colony of *Ae. aegypti* would be required for the mass production of *Tx. r. rutilus.* Unfortunately, *Toxorhynchites* larvae did not consume a non-living diet. If it was reared on a non-living diet, it required the longer development

time (Fock *et al.*, 1978). However, there were pilot projects involving inundative releases of both eggs (Gerberg and Visser, 1978) and adults (Focks *et al.*, 1982) which indicated promise for the potential use of *Toxorhynchites* in biological control programs. Eventhough, there was interest in Laboratory rearing of *Toxorhynchites* mosquitoes (Focks *et al.*, 1977; Focks and Boston, 1979; Annis and Rusmiarto, 1988), the low yield of mosquito larvae and pupae were achieved. The reason for most larval mortality was due to cannibalism (Rubio and Ayesta, 1984). The first larval instar usually showed cannibalistic behavior even in presence of adequate number of prey. The fourth instar larvae were the most aggressive and even killed the prey larvae without eating them (Chowanadisai *et al.*, 1984). In Thailand, preliminary investigation on ecology of *Toxorhynchites splendens* was done and the population fluctuation as well as interaction between predator and prey were reported (Yasuno and Tonn, 1970). It could be argued that the effect of the predator larvae was to reduce the density of *Aedes aegypti* in containers without eliminating them completely. Such an effect was observed in field experiments (Fock *et al.*, 1982). *Toxorhynchites* larvae were poor control agents due to their inability to withstand periods of starvation and to their accidental removal from containers during the act of water consumption (Annis *et al.*, 1989). Since *Toxorhynchites* larvae were relatively slow moving and spent most of the time floating at the surface of the water. Disadvantage of *Toxorhynchites* was that their life cycles were

considerably longer than those of their prey, *Toxorhynchites splendens* had mean duration of a generation of 44.44 days (Chowanadisai *et al.*, 1984). Their life-cycle was 2-3 times longer than their prey so they could not respond quickly to changing mosquito densities. This might be associated with asynchronous egg development (Linley, 1987).

Bacteria

An increasing number of entomogenous strains of spore-forming bacteria were under investigation for the control of mosquito vectors of diseases. Two major bacterial insecticides, *Bacillus thuringiensis* serotype H-14 and *Bacillus sphaericus* strain 1593, had come to be recognized as the prime candidates for potential use since they had demonstrated high activity against a broad spectrum of mosquito larvae (Sebastien and Brust, 1981; Benjaphon *et al.*, 1987)

Bacillus thuringiensis was a gram-positive, aerobic, spore-forming bacterium that synthesized an intracellular crystalline inclusions during the sporulation cycle (Bechtel and Bulla, 1976; Bulla *et al.*, 1977). These inclusions consisted of protein exhibiting a highly specific insecticidal activity (reviewed in Aronson *et al.*, 1986). *Bacillus thuringiensis* crystalline inclusions dissolved in the larval midgut, releasing one or more insecticidal crystal proteins (also called δ-endotoxins) of 27 to 140 kilodaltons(kDa) (Höfte and

Whiteley, 1989). Most crystal proteins were protoxins that were proteolytically converted into smaller toxic polypeptides in the insect midgut. The activated toxin interacted with the midgut epithelium cells of susceptible insects and the toxins generated pores in the cell membrane, thus disturbing the osmotic balance. Consequently, the cells swelled and lysed. The larvae stopped feeding and eventually died. For several *B. thuringiensis* toxins, specific high-affinity binding sites had been demonstrated to exist on the midgut epithelium of susceptible insects (Höfmann *et al.*,1988). This could, at least in part, explain the extreme specificity of these proteins. *Bacillus thuringiensis* were introduced to use as insecticides but there was the variation in insect host range and the most obvious factors that might influence the host range of a crystal protein were (1) differences in the larval gut affecting the solubilization and/or processing efficiency of the protoxin and (2) the presence of specific toxin-binding sites (receptors) in the gut of different insects (Höfte and Whiteley, 1989).

The susceptibility of the mosquito species towards *Bacillus thuringiensis* indicated that *Ae. aegypti* was found to be the most susceptible followed by *Culex quinquefasciatus* and slightly less susceptible by *Anopheles sp.* (Foo and Yap, 1982; Wongsiri and Andre, 1984). Due to its specificity, *Bacillus thuringiensis israelensis (Bti)* did not show a single case of human toxicity after over 23 years of operational use (cited in Margalit and Dean, 1985). The Informal

Consultation Group on Mammalian Safety of Microbial Control Agents for Vector Control, concluded in 1980 that the organism passed the necessary safety tests to warrant its application in large-scale field trials. In subsequent experiments negative results were obtained when *Bti* endotoxin was tested for mutagenicity *in vitro*. Maximum dosages were applied by the conventional oral, parenteral, respiratory and dermal routes, together with allergenicity tests and a mutagenicity screen; all confirmed that *Bti* posed no hazard. It could be concluded that *Bti* was safe, even for used in drinking water. However, it was found that when the *Bti* toxin in the crystal was dissolved in alkaline buffer, it became toxic, in very high concentrations, to suckling mice by intraperitoneal injection, but had no effect orally. Since in practice there was no chance that dissolved crystal which was sufficient to be harmful would reach the blood stream of man or mammal. *Bacillus thuringiensis* var. *israelensis* is now considered to be by far the safest mosquito larvicide (Margalit and Dean, 1985).

The major economic disadvantage of *Bti* was its low stability after application into the mosquito breeding habitats. The field performance of *Bti* was greatly influenced by the presence of organic matter or of solids in the water (Ignoffo *et al.*, 1981; van Essen and Hembree, 1982). They found that the rate of inactivation of *Bti* by organic material, as well as the adsorption of *Bti* on soil particles and on organic matters in the water greatly reduced its

persistence and its field efficacy. Although *Bacillus thuringiensis* did not show long residual effects (Lee and Cheong,1987), it could be used alternatively with chemical larvicides to delay the development of resistance in mosquito populations. Resistance to the spore-crystal protein complex of *Bacillus thuringiensis* has been presumed unlikely to occur. There have been few reports of insect resistance to microbial insecticides (McGaughey, 1985), leading to the presumption that insects are unlikely to become resistant to these agents (McGaughey, 1985). *Bacillus thuringiensis* var. *israelensis* is still relatively expensive but in the future the price of *Bti* may be reduced due to improved technology and an expanding market. The future of *Bti* will very likely involve genetic engineering. Specifically, it will be desirable to transfer the toxin gene(s) into a bacterium which would survive well and produce the toxin under the low organic conditions of most stagnant ponds. A few biological agents such as *Bacillus sphaericus* have been reported to survive well under such conditions but are not as toxic as *Bti*. Through the combined efforts of geneticists, microbiologists and insect pathologists, improved control of insect vectors will be forthcoming (cited in Margalit and Dean, 1985).

Bacillus sphaericus, another spore-forming bacterium, was also highly pathogenic for mosquito larvae. Though many strains of this organism were known. The toxicity of *B. sphaericus* strain 1593 was found to be greater towards *Cx. quinquefasciatus* larvae than

Ae. aegypti larvae, whereas the toxicity of *B. thuringiensis* serotype H-14 was found to be greater towards *Ae. aegypti* larvae than *Cx. quinquefasciatus* larvae (Silapanuntakul *et al.*, 1983). One of the important features of *B. sphaericus* strains was their relatively slow action against larvae compared with *Bacillus thuringiensis* H-14 (Mulla *et al.*, 1984). However, *B. sphaericus* 1593 had good persistence and the ability to recycle. Thus, it was ecologically more interesting (Silapanuntakul *et al.*, 1983). *Bacillus sphaericus* produced a toxin that was associated with the presporulating cell or the spore; toxin was not secreted into the medium. In the sporulating cell, the cell wall was the most toxic structure. However, the spore itself later developed a much higher level of activity (Myers and Yousten, 1980). The location of toxic activity in the spore has not yet been determined. Some insecticidal strain those that were highly toxic in the spore stage, produced parasporal inclusions that resemble the crystals of *B. thuringiensis*. The increase in toxicity of these cells during sporulation was paralleled by the development of crystal like inclusions, which were rapidly dissolved in the larval gut (Yousten and Davidson, 1982). As these inclusions have not yet been isolated, their toxicity is not known. Both *Bacillus thuringiensis israelensis* H-14 and *Bacillus sphaericus* were microbial insecticides acting as a stomach poison but these species did not persist in the environment. Moreover, suspended particulate matter, organic and inorganic pollutants reduced its

efficiency, and in deep waters it tended to settle down amongst bottom sediments so that little was ingested by mosquito larvae (cited in Service, 1983).

2. Fungal pathogens of mosquitoes

Idea of using fungi to control insects of medical and veterinary importance did not receive wide consideration until the advent of biological control concepts in recent years. There were a wide range of taxonomically different fungi caused pathogenesis in mosquitoes such as *Coelomomyces*, *Lagenidium*, *Culicinomyces*, *Metarhizium* and *Tolypocladium* etc. which were introduced to use as a potential biological control agents.

Coelomomyces

Coelomomyces (Class Chytridiomycetes, Order Blastocladiales), of which there were about 40 reported species, was an aggressive parasite of mosquito larvae throughout the world and was potentially important because of its capacity to parasitize *Anopheles* and *Aedes sp.*, the main vectors of disease. It was first reported in 1921 but only in the last decade has its life-cycle been elucidated, and this depended on the chance discovery that a large degree of parasitism of mosquito larvae required the presence of other animals - the

small copepods, like *Cyclops*. *Coelomomyces* has, in fact, an obligate alternation of hosts: the spores released from mosquitoes can infect only copepods, and *vice-versa* (Whisler *et al.*, 1975). Dissection of the *Cyclops* indicates that planonts (refered to flagellated swarm cells) are cleaved out of wall-less thalli which have developed in the haemocoel of the host animal. Then, they escape to the external environment through a tear in the host's integument. The infective planonts from the copepod has either one or two posteriorly inserted flagella and their general morphology resemble that of the zoospores from resting sporangium of *Coelomomyces*. The planonts with a single flagellum are uninucleate, whereas, the biflagellate planonts contains either one or two nuclei. Explanation of this nuclear difference is that, sexual reproduction, involving uniflagellate isogametes fused to form a biflagellate zygote. Infected copepods release both gametes and zygotes but the gametes will not infect the larvae. Mosquito larvae are infected by a zygote with a diploid nucleus (Figure 1). Such an obligate alternation of hosts is evidence of a high degree of specialization in parasitism. Another interesting feature of *Coelomomyces* is its mode of infection. When a zoospore or motile zygote landed on the host surface (usually in an intersegmental region) it encysted, germinated to form an appressorium and then formed a thin penetration tube. The spore protoplasm was then injected into the host by the development of a large vacuole in the spore, and the fungus grew as a naked

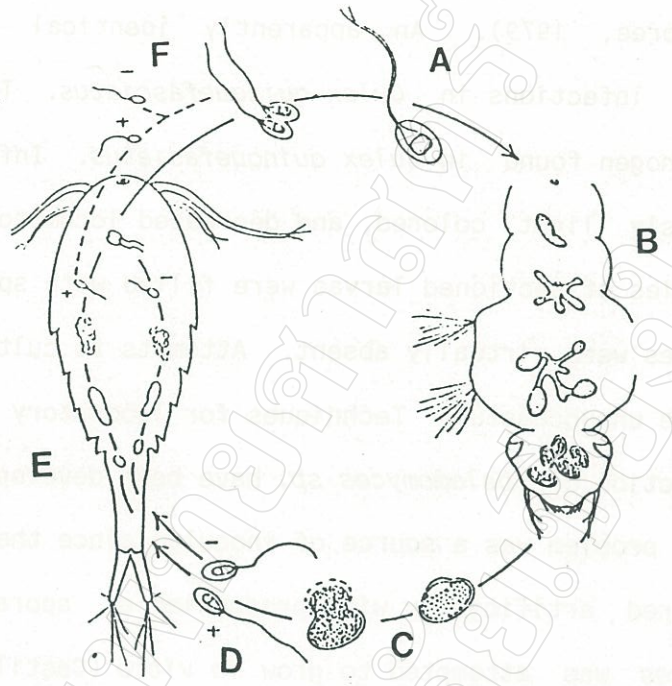

Figure 1. Life cycle of *Coelomomyces psorophorae*. Zygote (A) infects larva of *Culiseta inornata* (B) leading to development of hyphal bodies, mycelium and, ultimately, thick-walled resistant sporangia. Under appropriate conditions these sporangia (C) release zoospores of opposite mating type (D) which infect the alternate host, *Cyclops vernalis* (E). Each zoospore develops into a thallus and, eventually, gametangia. Gametes of opposite mating type (F) fuse either in or outside of the copepod to form the mosquito-infecting zygote. (Whisler, H.C. *et al.*: Proc.Nat.Acad.Sci.USA 72: 695, 1975)

protoplasmic mass in the host body cavity (Deacon, 1984). *Coelomomyces sp.* was seen in *Aedes aegypti* from Chiang Mai, Lampang etc. (Hembree, 1979). An apparently identical organism caused septicemic infections in *Culex quinquefasciatus.* This was the most common pathogen found in *Culex quinquefasiatus.* Infected larvae were conspicuously light colored, and decreased locomotory ability. The body cavities of sectioned larvae were filled with sporangia, and fat body tissues were virtually absent. Attempts to culture it in various media were unsuccessful. Techniques for laboratory infection and *in vivo* production of *Coelomomyces sp.* have been developed (Couch, 1972). The main problem was a source of inoculum since the fungus has not been cultured artificially with production of sporangia. However, *Coelomomyces* was attempted to grow *in vitro* (Castillo and Roberts, 1980). Significant growth of hyphal fragments and differentiation into young resting sporangia occured in conditioned Mitsuhashi-Maramorosch insect tissue culture medium but these sporangia did not produce zoospores and *Coelomomyces* could not longterm *in vitro* culture, and this was one of the potential limitations to its use as a biological control agent.

Culicinomyces clavosporus

The genus *Culicinomyces* (Class Deuteromycetes, Fungi Imperfacti) consists of two isolates of a facultative parasite of

mosquitoes. The type species, *C. clavosporus* (Couch *et al.*, 1974) was isolated from a laboratory colony of *Anopheles quadrimaculatus* in the USA although another isolate with similar properties was isolated from a colony of *An. amictus hilli* in Australia. *Culicinomyces clavosporus* develops hyaline, septate, branched hyphae and forms conidiophores, which might be straight and unbranched or much elongated and branched; first conidium apical, attached to the flask-shaped mother cell by a narrow tube; conidiophores branch and elongate forming conidia in verticles or in lateral or terminal penicillate like structures arising from the main conidiophore stalk. Primary conidia larger than later form ones, hyaline, club shaped and nonseptate (Couch *et al.*, 1974). The usual route of fungal infection was *via* the exocuticle, however, in the mosquito pathogenic fungus *Culicinomyces clavosporus*, the primary route of infection was through the foregut and hindgut following ingestion of the conidia (Sweeney, 1975). The sites of infection were the pharynx and oesophagus as well as the ileum, colon and rectum of the hindgut, on rare occasions, the walls of the preoral cavity. Infection was not recorded from the midgut or outer integument. The conidia are long ovoid, narrow at the apex and broader at the base. They have been observed adhering to the cuticle of all the infection site of the gut before showing any visible signs of germination. Germination always occurs at the narrower end; a slight swelling developes from which a short germ tube grow. The tip of the germ tube appeares to be the only area of the

spore in intimate contact with the cuticle. The integument is pierced by a slender penetration hypha which extends through the gut epithelium unchanged in size until it reaches the haemocoel, where it swells into an oval, sometimes almost spherical, vessicle. The time taken for infection to establish in the haemocoel is varied from 16 hours to 4 days. After successful penetration, hyphae branches and radiates out into the coelom. Hyphal bodies bud off laterally and circulate in the haemolymph; some germinates to form short hyphal strands. Some mosquitoes died shortly after the penetration stage or during early hyphal growth in the haemocoel. However, most infected mosquitoes did not die until densely packed hyphae almost completely filled the body cavity.

The activity of the Australian and United States strains of *C. clavosporus* was compared and it was found that there was no difference in potency of the two strains. Its host range included several species of mosquitoes but *Aedes aegypti* was more susceptible (Cooper and Sweeney, 1982). It was demonstrated that *Culicinomyces clavosporus* killed *Chaoborus sp.* and *Chiromomus sp.* (Knight, 1980). On artificial media the optimum temperature for conidia germination was 27.5°C and the optimum for growth was 25°C; at 30°C germination took place but growth did not ensue. With mosquito larvae, good infection rates and kills were observed between 15°C and 27.5°C but very little at 30°C (Knight, 1980). One of the limitations of *Culicinomyces* as a biocontrol agent was its apparent inability to

demonstrate significant persistance and recycle after application to the field (Sweeney, 1981; Sweeney *et al.*, 1983).

Tolypocladium cylindrosporum

Tolypocladium cylindrosporum (Deuteromycotina: Moniliales) is originally described as a soil saprobe in Europe and one isolate from Northern California was sequently found parasitising larvae of the treehole mosquito *Aedes sierrensis*. Almost a decade earlier Pillai had isolated another strain of this fungus from larvae of the mosquito *Ae. australis*, which bred in hypersaline supralittoral pools along the southern coast of the South Island of New Zealand (cited in Federici *et al.*, 1980; Gardner and Pillai, 1986). *T. cylindrosporum* as many deuteromycetes were easily grown in artificial media (Gardner and Pillai 1986, 1987) where it formed conidia on agar media and blastospores in submerged culture such as this fungus produced abundant mycelium in 5-10 days and sporulation occured after 7 days when it was grown on Sabouraud's dextrose agar plates, whereas, this fungus produced infective blastospores in Sabouraud's dextrose broth and in cheap and more readily available media such as media derived from coconuts (Gardner and Pillai, 1987). However, there was no apparent difference between the virulence of the two spores types. The mean 50 percent lethal concentration (LC50) was approximately $2x10^5$ spores/ml against *Ae. australis* but blastospores were found to

penetrate and kill larvae much more rapidly than conidia, the 50 percent lethal time (LT 50) for blastospores was 3.8 days whereas for conidia was 7.9 days (Gardner and Pillai, 1987). This difference was probably due to differences in germination time.

The mode of infection and cycle of *T. cylindrosporum* was examined in *Aedes sierrensis* and *Culex trasalis* (Soares, 1982). Larvae were found to be infected through the external cuticle, the buccal cavity and phyrynx, and the midgut. Penetration appeared to occur randomly on the external cuticle particularly for blastospores which readily attached to the integument. Sites of penetration could often be defected macroscopically by the presence of melanized areas on the integument, where frequently revealed a localized mycosis. Localized infections with and without melanization were frequently observed on the abdomen and anal papillae. Infections localized in the head were often noted to originate in the buccal cavity and pharynx. Nevertheless, germinating conidia and blastospores could penetrate the peritrophic membrane and many hyphae could be seen penetrating through the midgut epithelium into the haemocoel. Host reaction to *Tolypocladium* appeared by encapsulation and melanization of invading hyphae. Infected larvae generally did not die until they were heavily infected by the fungus and hyphae packed a major portion of the body. After a larva was killed and the haemocoel became packed with mycelium, hyphae began to emerge through the outer cuticle. Secretion of enzymes and digestion of the cuticle at the point of

contact with the hypha appeared to be the principle factor in penetration. After the hyphae penetrated out of the cadaver, they continued to grow. If the cadaver was submerged the hyphae would grow outward in all directions from all parts of the body. Such growth continued until the nutrients in the cadaver apparently were exhausted. Neither conidia nor blastospores were produced on these submerged hyphae. If the cadaver was near the surface of the water, the hyphae would grow to the surface and sporulate.

The pathogenic potential of this fungus has also been demonstrated against various species of mosquito host. *Culex tarsalis* larvae were less susceptible to *T. cylindrosporum* infections than *Aedes sierrensis.* The greater resistance of *Cx. tarsalis* correlated well with evidence of more effective humoral encapsulation of invading hyphae relative to *Ae. sierrensis* (Soares, 1982). *Mansonia uniformis* had high susceptibility towards *T. cylindrosporum.* It seems to indicate that this fungus is quite promising as a biocontrol agent for this mosquito species. Besides, *Mansonia sp.* had a considerably long larval period which could provide sufficient time for the fungus to act. Because this fungus seems to be slow acting and it required a longer time to develop in the gut of its host but, for *Ae. aegypti*, this fungus appeared to be less effective (Serit and Yap., 1982).

Tolypocladium cylindrosporum (The New Zealand strain) exhibited good tolerances to temperature (4-35°C), pH (3-10) and salinity (0-7% NaCl). It was able to survive conditions up to 7%

NaCl which was approximately twice the salt concentration of seawater. The North American isolate of the fungus showed similar tolerances, while the European isolate was less tolerant (Gardner and Pillai, 1986). These provided essential background information about the environment tolerances of this pathogen of mosquito larvae. The results obtained, particularly the temperature and salinity tolerances of the fungus, appeared to the wide-ranging. Preliminary field trials with *T. cylindrosporum* was introduced in central Alberta and five species of mosquitoes were found infected following application of blastoconidia (Goettel, 1987). Introduction of conidia into crab holes in Fiji resulted in reductions of 87% of immature *Aedes polynesiensis* (Gardner *et al.*, 1986), but application of blastospores to ground pools in New Zealand were not as successful (Gardner and Pillai, 1987). The failure might be due to fluctuations in the physical conditions and the low water temperature at the lowest extreme for the activity of this fungus which could influence and limit the biological activity of this pathogen in the natural habitat. Unfortunately spores of *T. cylindrosporum* were highly vulnerable to desiccation and this might be a limiting factor to their use (Gardner and Pillai, 1987). Besides, the pathogenicity of *T. cylindrosporum* demonstrated against some of the non-target organisms such as diphnid, dixid larvae and copepods (Gardner and Pillai, 1987) could also present a risk, the future of this agent as a potential mosquito larvicide remains uncertain. This non-specificity may be a problem that hinders

the use of this and other deuteromycetous fungi in aquatic ecosystems.

Metarhizium anisopliae

Metarhizium anisophiae (Class Deuteromycetes, Fungi Imperfecti (Hyphomycetes), Order Moniliales) is separated in two varieties: var. *anisopliae* for the short-spored taxon (6-8 μ) and var. *major* for the long-spored taxon (9-14 μ). Both varieties show a wide range of greens. The fungus causes disease in insects, the syndrome known as green "muscardine" (World Health Organization, 1980). *Metarhizium anisopliae* is a facultative parasitic fungus which can grow either as a parasite of insects or as a saprophyte. However, this fungus was found as a possible mosquito control agent (Romoska *et al.*, 1981). The life cycle of *M. anisopliae* is typical of imperfect fungi, with asexual reproduction resulting in spores borne on hyphae (conidia). On terrestial insects, development begins by penetration of the cuticle, either directly by germ tubes from condidia or by means of infection pegs produced from buttom- like structures (appressoria) which develop at the end of short germ tubes. Development then takes place in the hemocoel, usually as yeast-like hyphal bodies. The insect is killed by toxins followed by invasion of the organs by mycelium. The insect may remain a sclerotium if the microclimate is dry. In warm, moist conditions, conidiophores may be extended through the cuticle to cover the insect with conidia.

However, the insect-pathogenic members of the Deuteromycotina can cause major damage to insect populations. Nevertheless, epidemics are again weather-dependent; a high relative humidity is always required in the initial stages of penetration of the cuticle from spores, although the subsequent course of infection is not humidity-dependent (World Health Organization, 1980; Deacon, 1984). The cycle in mosquito larvae is different from the above. The principle mode of action in most cases is obstruction of the air passage through the two tracheal trunks of the larva. *Metarhizium anisopliae* conidia adhere to the perispiracular valves, germinate and penetrate through the cuticle into the hemocoel. The spiracles become obstructed and the lack of air and perhaps toxins produced by the fungus in the hemolymph causes death of the host (World Health Organization, 1980). After host death, the fungus grow throughout the insect body in what is termed the "saprophytic" phase of growth, because *M. anisopliae* had antibiotic properties against the common saprophytes such as *Aspergillus, Cladosporium, Penicillium, Rhizopus* and *Trichoderma* (Walstad *et al.*, 1970) and might therefore exclude potential competitions from the cadaver. Floating spores, when ingested, can apparently without germination release lethal substances into the gut. This is much less frequently observed than perispiracular siphon infection (Cited in World Health Organization, 1980). *Metarhizium anisopliae* produces a family of toxins called destruxins and other compounds such as cytoclalasin D and E which probably toxic to

mosquito larvae. Destruxins have not been detected in extracts of conidia but was found in living mosquito larvae following exposure to conidia (Cited in World Health Organization, 1980). As a result, sporulation does not take place on the external cuticle, and therefore *Metarhizium* does not have the potential to recycle in larval populations and it has a very wide natural range of insect hosts, so for environmental reasons it is less attractive as a mosquito larvicide than some of the other more specific microbial agents. Nevertheless, it does have some favorable characteristics. The conidia have a long shelf life and are readily produced in semisolid media using simple technology, including no infections of warm-blooded animals have been reported and there are no reports of human sensitivity to *M. anisopliae* (Reviewed by World Health Organization, 1980; Davidson and Sweeney, 1983).

Lagenidium giganteum

Lagenidium giganteum (Class Oomycetes, Order Lagenidiales) is a facultative parasitic fungus which can grow vegatatively either as a saprophyte in the aquatic environment or as a parasite of mosquito larvae. A natural epizootic of the fungus *Lagenidium giganteum* in larval populations of *Culex tarritans* was also reported (Glenn and Chapman, 1978). Its life cycle is typical of the genus *Lagenidium* with both sexual and asexual reproduction (McCray, 1985). The infective form of *Lagenidium* is the biflagellate motile zoospore,

and the two distinct portals of entry for mosquito are the mouth and the cuticle. In the majority of the infected larvae, zoospores are concentrated by the mouth parts and penetrate the tissue of the host larvae in the anterior portion of the digestive tract, usually in the region of the pharynx. Mycelial growth spreads rapidly from the point of entry throughout the head, then *via* the haemocoele toward the posterior, infecting the anal segment and anal gills last. However, in about 2% of the specimens observed, mycelial growth first appeared in the abdomen, anal segment or anal gills. In these larvae the hyphae grew from darkly pigmented spots on the chitinous exoskeleton. Numerous larvae were observed with infections occuring through both portals of entry. The hyphae which grow in the haemocoele spread rapidly throughout the body, and then bocome septate and enter their asexual reproductive phase (Figure 2). The individual segments of the hyphae become rounded, granular structures known as sporangia and sporangial formation and larval death are usually simultaneous. A fourth stage larva will usually contain about 20,000 sporangia. One, sometimes two, exit tubes are then formed by each sporangium and grow out the body wall of the dead larva. A vesicle is formed at the end of each exit tube, and the entire contents of the sporangium flow into the visicle. Within the vesicle are formed numerous zoospores (average of 12/vesicle). The wall of the vesicle disintegrates and the motile zoospores are released into the water to repeat the cycle. Zoospores are ovoid, on one side of the zoospore, a groove run from

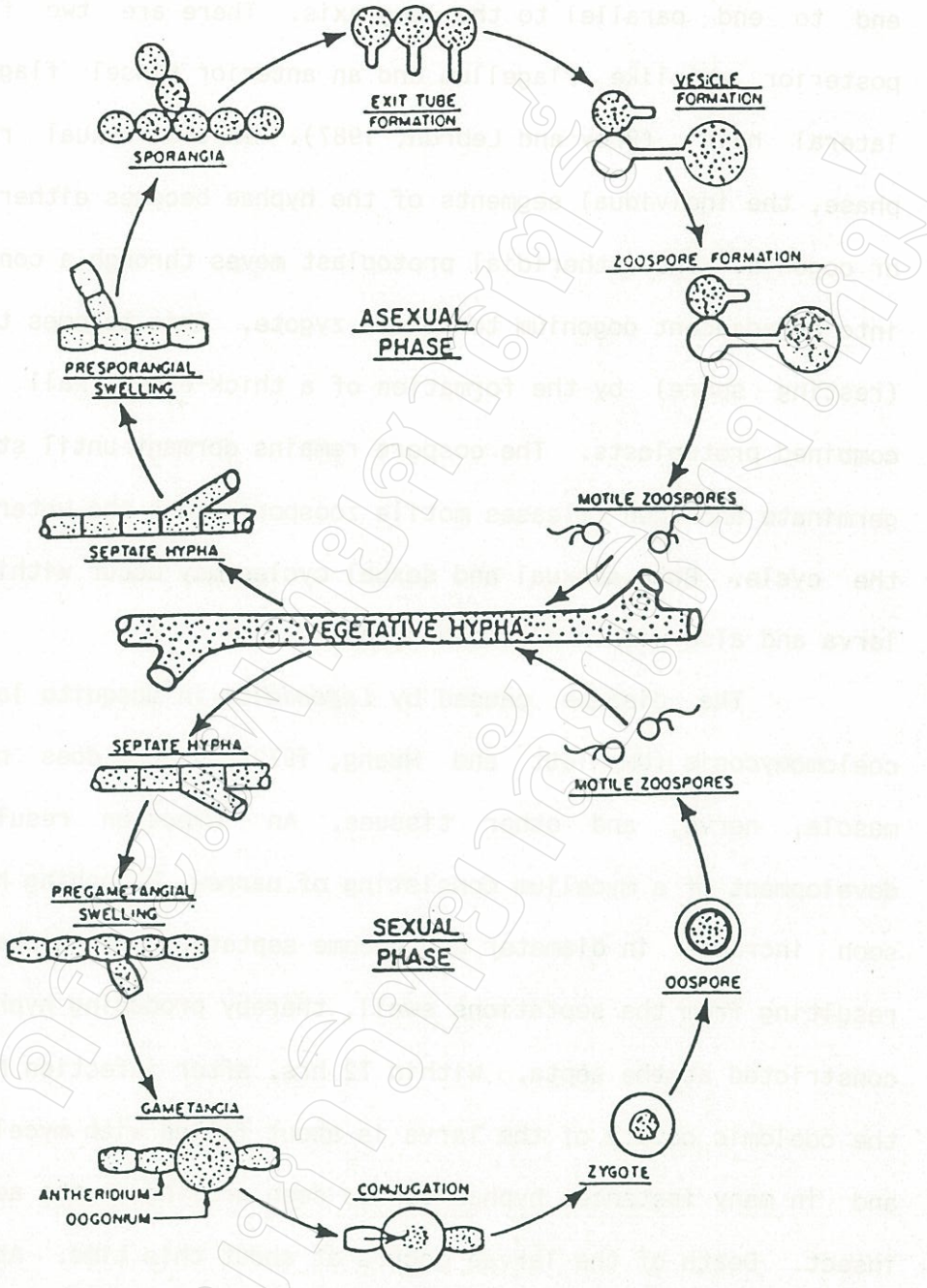

Figure 2. Life cycle of *Lagenidium giganteum.*

(McCray, E.M., Jr.: Am. Mosq. Control Assoc. Bull. No.6: 88, 1985)

end to end parallel to the long axis. There are two flagella, a posterior whiplike flagellum and an anterior tinsel flagellum with lateral hair (Brey and Lebrun, 1987). In the sexual reproductive phase, the individual segments of the hyphae becomes either antheridia or oogonia. The antheridial protoplast moves through a conjugal pore into an adjacent oogonium to form a zygote. This becomes the oospores (resting spore) by the formation of a thick-ended wall around the combined protoplasts. The oospore remains dormant until stimulated to germinate and then releases motile zoospores into the water to repeat the cycle. Both asexual and sexual cycles may occur within a single larva and also within a single hypha.

The disease caused by *Lagenidium* in mosquito larvae is a coelomomycosis (Umphlett and Huang, 1972) which does not involve muscle, nerve, and other tissues. An infection results in the development of a mycelium consisting of narrow, branching hyphae which soon increase in diameter and become septate. The hyphal segments resulting from the septations swell, thereby producing hyphae that are constricted at the septa. Within 72 hrs. after infection has occured, the coelomic cavity of the larva is about filled with mycelial growth, and in many instances hyphae can be seen growing in the aorta of the insect. Death of the larvae occurs at about this time. About 24 hrs. after an infected larva is dead, zoospore production is initiated. The hyphal segments produce thin discharge tubes that penetrate the exoskeleton of the dead larva. Through the tubes the cytoplasm

contained in the segments is discharged to the outside where it is retained for a few minutes in a membranous vesicle. Cleavage of the cytoplasm occurs in the vesicle, and the biflagellate zoospores formed there escape when the vesicle breaks down.

Lagenidium giganteum can be grown *in vitro* on a variety of solid or liquid media (Domnas *et al.*, 1982; Jaronski *et al.*, 1983; Jaronski and Axtell, 1984; Kerwin *et al.*, 1986). The use of *L. giganteum* for biological control of mosquitoes requires a simple and rapid means of mass-producing infective zoospores. All earlier field evaluations of *L. giganteum* have used this fungus reared in living mosquito larvae (e.g., Umphlett and Huang, 1972). Such methods are time consuming, labor intensive, and pose problems in the quantification of doses. This organism required sterols for induction of sexual reproduction (Kerwin and Washino, 1983) and for zoospore formation (Domnas *et al.*, 1977). But increasing concentrations of glucose or peptone improved mycelial yield but repressed zoospore output, and there was no proportional increase in zoospores with increasing mycelial yield (Domnas *et al.*, 1982).

The effectiveness of *L. giganteum* under field conditions were evaluated (Jaronski and Axtell, 1983; Kerwin and Washino, 1986; Guzman and Axtell, 1987). Several studies have shown that the fungal pathogen *Lagenidium giganteum* can persist in mosquito breeding habitats for prolong periods (Glenn and Chapman, 1978; Jaronski and Axtell, 1983) and recycle for the entire season despite periodic

scarcity of hosts and short-term drought (Jaronski and Axtell, 1983; Kerwin and Washino, 1986). Oospores of *L. giganteum* are responsible for the survival of the fungus. Several oomycetous fungi have shown potential as microbial control agents for mosquito larvae. Unfortunately, the most promising among them, *Lagenidium giganteum,* cannot infect larvae in water with greater than 1 ppt (part per thousand) of NaCl (Merriam and Axtell, 1982). In water containing 1.5 ppt NaCl, there was complete inhibition of zoosporogenesis and mosquito infection. Thus, it was unsuitable for use as biocontrol agents for mosquitoes in saline habitats. The influence of temperature on the success of *L. giganteum* in limited field experiment was reported (Jaronski and Axtell, 1982) and highest mosquito larval infection rates occurred at 21-29°C (Jaronski and Axtell, 1983). The low temperatures caused a longer development time of *L. giganteum* in the infected hosts (Guzman and Axtell, 1987). This fungus was an ineffective biological control agent in mosquito breeding habitats polluted by organic matter because the effects of polluted water were to reduce or block zoosporogenesis in *L. giganteum* (Jaronski and Axtell, 1982). However, it was effective in controlling mosquito larvae in unpolluted pond water.

Leptolegnia sp.

Leptolegnia sp. (Oomycetes: Saprolegniales) is an aquatic

fungus with a filamentous. eucarpic, non-septate thallus. The genus is described as having hyphae that are long, slender, delicate, sparingly branched and have a wall composed largely of cellulose and β-glucan. Long, straight sporangia are formed from the filamentous undifferentiated, vegetative hyphae. Spores, formed in a single row within the sporangia (zoosporangia) are elongate on discharge, form an oval shape and swim immediately after discharge from a pore at the tip of the sporangia (Alexopoulos and Mims, 1979; Seymour, 1984; Akpan Eyo, 1987). These swimming spores (zoospores) are dimorphic and biflagellated. Upon emergence from zoosporangia, the motile primary zoospores become pear-shaped with apical flagella, and swim for a period of time before encysting to from a big round primary spore. After a few minutes, the encysted primary spores emerge as kidney-shaped secondary motile zoospores and swim for a longer period before encysting into another round-shaped spore smaller in size than the encysted primary spores. This fungus exhibits both asexual and sexual reproductive patterns. Asexual reproductive involves encystment and germination of zoospores released from cylindrical, terminally located zoosporangia. Sexual reproduction in known species of Oomycetes fungi involves gametangial contact preceded by meiosis, which occurs in the gametangia, and is followed by formation of zygotes (oospores) (Alexopoulos and Mims, 1979; Seymour, 1984; Akpan Eyo, 1987).

Leptolegnia sp. is a virulent pathogen whose host range

appears to be restricted to mosquitoes (McInnis *et al.*, 1985). McInnis and Zattau (McInnis and Zattau, 1982; Zattau and McInnis, 1987) identified two methods by which the zoospores infect their hosts, one by cuticular encystment of secondary zoospores, and two by germination of zoospore cysts in the alimentary canal. These is similar to another experiment (Nnakumusana and Seymour, 1981) which found that the zoospores encysted on the cuticle of heat killed mosquito larvae under phase contrast light microscopy. When live larvae were dissected after exposure, the gut was found packed with zoospores. Hence, *Leptolegnia sp.* caused mosquito larvae infection by encystment on and penetration of the cuticle or *via* the gut (Lord and Fukuda, 1988). Symptoms of infection include excessive grooming, gaping of mouthparts, palpitation, change in color and position, cessation of feeding, reduction in wiggling, eventual stuggishness and death. Infected larvae died from: (a) cytological damage to tissues in infected organs, (b) suffocation from encysted spores blocking the tracheae, (c) destruction of hemocytes and fat bodies (Akpan Eyo, 1987) and this fungus induced larval mortality quite quickly, 78-96% mortality within 24 hours, and 100% mortality within 48 to 72 hours. Host range studies are carried out on *Leptolegnia sp.* which has a broad host range in the aquatic systems. It could infect mosquito larvae of *Aedes aegypti, Culex pipiens pipiens, Anopheles quadrimaculatus, Toxorhynchites rutilus,* etc. (Nnakumusara and Seymour, 1981; Nnakumusara, 1986), however, zoospores of this fungus

did not encyst or infect frog eggs, tadpoles, gypyfish, the blue gill fished and *Cyclops*. Although no successful field infection has yet been documented but *Leptolegnia sp.* exhibited potential as a mosquito larvicides. Furthermore, *Leptolegnia chapmanii* which was isolated from *Culex* larva collected from a ground pool at the edge of a salt marsh could infect mosquito larvae in water with greater than 5 ppt (part per thousand) of NaCl (Lord *et al.*, 1988). This fungus infected mosquitoes in water with NaCl concentrations well above the tolerance limits of *Lagenidium giganteum.* However, one disadvantage noted was that *Leptolegnia* killed 100 percent *Toxorhynchites brevipalpis* (Nnakumusana, 1986) which are useful for their predatory habit on mosquito larvae of other genera. It would be an added advantage if this fungus could be integrated with the predator to keep the mosquito larval population down.

III. MATERIALS AND METHODS

A. Isolation and screening procedures

1. Collection of mosquito larvae

Mosquito larvae samples were collected from 100 various habitats in the northern Thailand provinces during field visits. After recording about places, mosquitoes' genus, temperature and pH of water, they were collected in microtiter plate and brought back to the laboratory for examination mosquito larvae's abnormalities.

2. Examination of fungi

Mosquito larvae samples were selected by observing abnormalities of movement and colour and then, they were examined with microscope to find hyphae or sporangium in them.

3. Isolation of fungi

Mosquito larvae were washed 2-3 times with 50 µg/ml Chloramphenicol in sterile distilled water. Then each of them was transferred to isolation media; cornmeal agar fortified with 1% dextrose and 0.2% peptone (CMDP) (Umphlett and Huang, 1972) and

peptone - yeast extract - glucose agar (PYG) consisted of 0.12% peptone, 0.12% yeast extract, 0.3% glucose and 1.5% agar in distilled water (Jaronski and Axtell, 1984). A pure culture of fungus was isolated and prepared for subsequent experiments.

B. Studies of mosquito larvae killing ability of fungi

1. Colonization of mosquito larvae

Culex quinquefasciatus, *Aedes aegypti*, *Anopheles dirus* and *Anopheles minimus* were colonized and maintained in the insectarium. All mosquitoes were indigenous to Thailand and were of medical importance. Late second or early third instar larvae were used for bioassays and being tested to free from fungal infection by microscope examination and culture.

2. Laboratory testing to determine mosquito larvae killing ability of fungi

2.1 Bioassays

The bioassays were carried out at first as screening procedures. The test fungus was cultured on cornmeal agar (Difco Laboratories, U.S.A.) fortified with 1% dextrose (Difco

laboratories, U.S.A.) and 0.2% peptone (Difco laboratories, U.S.A.) (CMDP) and stored for 7-14 days at room temperature (25-30°C) before being used in bioassays.

For the bioassay, 1 ml of spore suspension was added at an approximate concentration of 10^6 - 10^8 spores/ml to each of 20x100 mm petri dish containing 70 ml of sterile distilled water. At the same time 20 second to third instar larvae each of *Cx. quinquefasciatus*, *Ae. aegypti*, *An. dirus* or *An. minimus* were added to each petri dish and they were fed with sterile ground mosquito larvae food for a period of 5 days. This experiment was done in duplicate including control without fungal spores. The bioassay was conducted at room temperature.

Experiments were terminated after 5 days and the mortality after 1, 2, 3, 4 and 5 days fungal exposure were recorded. Larvae that were presumed dead were probed for possible movement with the rounded tip of a Pasteur pipette. Then, the dead larvae were examined for presence of characteristic hyphae or sporangia with microscope and cultured on media to confirm the identify of test fungus. The control was tested in the same manner.

The mosquito larvae killing ability was estimated by % mortality, as calculated by the equation below.

$$\%\text{mortality} = \frac{\text{no.of mosquito larvae died in test - no.mosquito larvae died in control}}{\text{total no. of mosquito larvae used}} \times 100$$

If mosquito larvae killing ability of test fungus was more than 50% mortality, next experiment would be performed to determine the lethal concentration (LC 50).

2.2 Determining the lethal concentration (LC 50)

Experiments were conducted in the same way as screening test but mosquito larvae were exposed to various concentrations of fungal spores. The bioassays were performed at concentrations of 10^3, 10^4, 10^5, 10^6 and 10^7 spores/ml. Each concentration was done in duplicate and control without fungal spores.

Experiments were terminated after 5 days exposure and the mortality data for each species of mosquito larvae were summed up. The LC 50 values were determined as described by Reed and Muench (1938) (Cruickshank *et al.*, 1975)

C. Studies to morphology and optimal conditions for growth of mosquito larvae killing fungi.

1. Morphological studies

Examination of fungal morphology by macroscopic examination was done after each of mosquito larvae killing fungi had been cultured on different media. Texture, colour and size of the

colony on varying period of time were noted. This experiment was made at room temperature. At the same time, microscopic study was performed by wet mount with lactophenol cotton blue and slide culture method

Fungal growth and spores production were examined when the fungus was cultured on Sabourand's dextrose agar (Difco laboratories, U.S.A.), Potato dextrose agar (Difco laboratories, U.S.A.), peptone - yeast extract - glucose agar (PYG) that consisted of 0.12% peptone (Difco laboratories, U.S.A.), 0.12% yeast extract, 0.3% glucose (Difco laboratories, U.S.A.) and 1.5% agar (Difco laboratories, U.S.A.) and the last medium was cornmeal agar (Difco laboratories, U.S.A) fortified with 1% dextrose and 0.2% peptone (CMDP). Fungal growth was measured by 2 different methods. The first method was done by measuring diameter of fungal colony after the tested fungal spore suspension had been inoculated on each kind of medium by using 0.01 ml standard loop. The test fungus was allowed to grow at room temperature and diameter of colony was measured at time interval. The experiment was made in triplicate.

The second method was done by measuring dry weight after the tested fungal spore suspension had been inoculated on sterile membrane filter paper, pore size 0.2 um, 45 mm. in diameter that placed on each media. The fungus was allowed to grow until the colony nearly covered the filter paper. Then, the filter paper was pulled out and let it dry in 37 °C incubator before the dry weight was measured with

analytical balance.

Spores production of test fungus, cultured on various media was measured by using hemocytometer chamber. Spores was counted with medium power objective (x40) after the test fungus had been allowed to grow 3, 5, 7, 14 and 24 days on slant media. Five milliliters each of sterile distilled water (with 0.05% Tween) was added to slant cultures then mixed throughly. This experiment was made in duplicate.

2. Studies of optimal conditions for growths of mosquito larvae killing fungi.

2.1 Temperature

Temperature effect on fungal growth was determined when the test fungus was cultured on the same media of C-1 and the same inoculum size was inoculated with 0.01 ml standard loop on agar plate to measure diameter of fungal colony and on slant media for spore enumeration as described in C-1. Fungal cultures were held at different temperatures (10, 20, 25 and 37°C). Diameter of fungal colony was measured daily and spores production was counted at day 3, 5, 7, 14 and 24.

2.2 pH

The test fungus was cultured on media which were adjusted pH to pH 4, pH 5, pH 6, pH 7 and pH 8 with hydrochloric acid (May & Baker LTD. Dagenham England) and sodium hydroxide (MERCK) in Michaelis' Veronal Acetate buffer. This buffer consisted of sodium acetate, sodium diethylbarbiturate (RIEDEL - DE HAËN AG SEELZE - HANNOVER) and 0.1 N HCl(See appendix). Any test fungi which could not grow on media prepared with Michaelis' Veronal Acetate buffer, would grow in those prepared with distilled water. Desirable pH was adjusted with HCl and NaOH after media had been sterilised by autoclaving at 112°C for 15 minutes.

Fungal growth and spore production were observed after the test fungus had been cultured on media with different pH. Colonial diameter and spore enumeration were subsequently determined as described before.

2.3 Nutritional requirements

2.3.1 Carbon assimilation test (Philpot, 1977)

Carbon assimilation tests were performed to determine appropriate sources of nutrients for growth of test fungi.

The basal medium consisted of 1.0 g potassium dihydrogen phosphate (Fluka AG, Chem Fabrik CH-9470 Buchs), 0.5 g magnesium sulphate 7 H_2O (Merck), 5.0 g ammonium sulphate (Riedel-de Haen AG Sulze-Hannover, Garmany) and 15.0 g agar (Difco Laboratories, U.S.A>) made up to 1 litre. A growth supplement was added to the medium before autoclaving at 112 C for 15 minutes. This supplement consisted of 1.0 mg thiamine (Sigma chemical company, U.S.A.), 250 mg i-inositol (Sigma chemical company, U.S.A.), 10 mg nicotinic acid (Fluka Chemie AG, CH-9470 Buchs) and 150 mg histidine (Fluka Chemie AG, CH-9470 Buchs) in 100 ml of distilled water. A 2 ml portion of this solution was added to each 100 ml of basal medium.

Carbon sources included the followings: glucose, galactose, maltose, lactose and saccharose (Difco Laboratories, U.S.A.). Sugars were prepared as 5% solutions and sterilised by filtration. The basal medium was made up to 80% of final volume and autoclaved twenty ml of the 5% carbohydrate was added to each 80 ml of the basal medium, to give a final concentration of 1% carbohydrate. The medium was dispensed aseptically into sterile plugged test tubes (20 x 150 mm) and sloped. Approximately 5 ml of medium was put into each tube.

Cultural procedures: Source of inocula for carbon assimilation studies were 7 days cultures of the test isolates at room temperature (26°C) on Hugh & Leifson (HL) agar that consisted of 0.5% sodium chloride (Riedel - de Haen AG Seelze-Hannover, Germany), 0.03% dipotassium hydrogen phosphate (Fulka Chemie AG, CH-9470 Buchs), 0.2%

peptone (Difco Laboratories, U.S.A.) an 1.5% agar (Difco Laboratories, U.S.A.). Small portions of mycelium were transferred by sterile inoculating needle to slopes of the compound under study. Growth was observed at 7, 14 and 21 days. The growth at 21 days was taken as the end reading. A control without added carbohydrate was included in each test.

2.3.3 Nitrogen assimilation test (Philpot, 1977)

The basal medium consisted of potassium dihydrogen phosphate (1.0 g), magnesium sulphate $7H_2O$ (0.5 g), glucose (10.0 g) ,agar (15.0 g) in 1 litre of distilled water, The growth supplement was added to this medium also before autoclaving. The medium was sterilised by autoclaving at 112°C for 15 minutes.

The nitrogen solutions were prepared to give quantities of nitrogen equivalent to 0.2% sodium nitrate (BDH Chemical Ltd. Poole, England), 0.26% asparagine (Difco Laboratories, U.S.A.), 0.24% ammonium sulphate (Riedel-de Haen AG Seelze-Hannover, Germany) 0.0225% urea (Merck) and 1% peptone (Difco Laboratories, U.S.A.). There were sterilised separately by autoclaving, excepted for urea and asparagine which were sterilised by filtration. The nitrogen solutions were added to the basal medium and the complete medium dispensed by pouring into 10x100 mm petri dishes.

Sources of inocula for nitrogen assimilation studies were 7 days cultures of test fungi on the nitrogen basal medium without a nitrogen source to exhaust any traces of nitrogen present in the inoculum. Fungi were then subcultured to nitrogen-containing media and growth was recorded at 7 and 14 days and the reading at 14 days taken as the end result. A nitrogen-free control was included.

The data were analysed by one-way analysis of variance excepted that the data determined by colonial diameter was analysed by the regression in groups (Armitage and Bery, 1987)

IV. RESULTS

A. Collection of mosquito larvae and isolation of fungi.

Mosquito larvae samples were collected from 1oo aquatic habitats in the northern Thailand provinces (Table 1). There were 12 provinces from which mosquito larvae were collected for fungal isolation. After microscopic examination, 49 mosquito larvae were found to be infected by fungi. The infection was 1.1 % of total mosquito larvae samples (4545 samples).

Of the total number of infected mosquito larvae , 38 (77.5%) were *Culex sp.* , 7(14.3%) and 4(8.2%) were *Anopheles sp.* and *Aedes sp.* respectively. Thirty two isolates of fungi were isolated from 49 infected cadavers (Table 2). These included 4 isolates of *Aspergillus niger* , 4 of *Penicillium sp.* , 3 of *Fusarium sp.* , 3 of *Trichoderma sp.* , 2 of *Cladosporium sp.* , 2 of *Paecilomyces sp.* and 1 each of *Curvularia sp.* , *Aspergillus niveus*, *Beauveria sp.*, *Aphanomyces sp.* and *Leptolegnia sp.* Nine isolates of fungi were unidentified.

B. Mosquito larvae killing ability tests of fungal isolates.

Mosquito larvae killing ability of fungal isolates was done against the same genus of mosquito larvae from which the fungal

Table 1 Collection of mosquito larvae from aquatic habitats in the northern Thailand provinces.

Province	number of collections
Phitsanulok	23
Chiang Mai	20
Chiang Rai	10
Lumpang	9
Mae Hong Son	8
Tak	8
Sukhothai	6
Lumphun	4
Phrae	4
Phayao	4
Uttaradit	2
Nan	2
Total number of collections	100 places

Table 2 Isolation of fungi from infected mosquito larvae collected from northern provinces.

Isolate number	Infected mosquito larvae	Province	fungi isolated
10A-15W	*Culex*	Chiang Mai	*Beauveria sp.*
12A-6	*Culex*	Chiang Mai	*Trichoderma viride*
16A-3	*Culex*	Lumpang	*Fusarium sp.*
16B-1	*Aedes*	Lumpang	*Fusarium sp.*
			Cladosporium sp.
16B-6	*Aedes*	Lumpang	Unidentify
16D-7	*Anopheles*	Lumpang	NG
16D-11	*Anopheles*	Lumpang	Unidentify
16D-13	*Anopheles*	Lumpang	Unidenfity
17A-3	*Culex*	Phitsanulok	NG
17B-5	*Culex*	Phitsanulok	*Cladosporium sp.*
17B-6	*Culex*	Phitsanulok	NG
17B-7	*Culex*	Phitsanulok	*Fusarium sp.*
20B-1	*Culex*	Mae Hong Son	*Aspergillus niger*
20B-2	*Culex*	Mae Hong Son	*Penicillium sp.*
20C-2	*Aedes*	Mae Hong Son	*Penicillium sp.*
21B-2G	*Culex*	Mae Hong Son	*Penicillium sp.*
23B-1	*Culex*	Lumpang	*Aspergillus niger*
23B-4	*Culex*	Lumpang	Unidentify
23B-6	*Culex*	Lumpang	NG
23B-8	*Culex*	Lumpang	NG
23B-10	*Culex*	Lumpang	Unidentify

Table 2 (Continued)

Isolate number	Infected mosquito larvae	Province	fungi isolated
23B-13	*Culex*	Lumpang	NG
23B-14	*Culex*	Lumpang	NG
23B-15	*Culex*	Lumpang	NG
26A-6	*Culex*	Phitsanulok	*Aspergillus niger*
26A-7	*Culex*	Phitsanulok	NG
26A-10W	*Culex*	Phitsanulok	Unidentify
26A-13Y	*Culex*	Phitsanulok	Unidentify
26A-15	*Culex*	Phitsanulok	*Aspergillus niger*
26A-19	*Culex*	Phitsanulok	NG
26A-21G	*Culex*	Phitsanulok	Unidentify
26D-7Y	*Culex*	Phitsanulok	*Paecilomyces sp.*
28C-4	*Culex*	Chiang Mai	NG
29B-5W	*Culex*	Nan	*Aspergillus niveus*
29F-2	*Culex*	Phrae	NG
29G-1	*Culex*	Phayao	*Curvularia sp.*
29G-2	*Culex*	Phayao	NG
29G-3	*Culex*	Phayao	*Trichoderma sp.*
29H-1	*Culex*	Phayao	NG
29I-1	*Anopheles*	Nan	*Trichoderma sp.*
29I-2Y	*Anopheles*	Nan	*Paecilomyces sp.*
29I-3	*Anopheles*	Nan	*Penicillium sp.*
29L-2	*Anopheles*	Phrae	NG

Table 2 (Continued)

Isolate number	Infected mosquito larvae	Province	fungi isolated
30B-1	*Culex*	Chiang Rai	Unidentify
30B-3	*Culex*	Chiang Rai	NG
35A-1	*Culex*	Sukhothai	NG
35A-3	*Culex*	Sukhothai	NG
35C-1	*Culex*	Sukhothai	*Aphanomyces sp.*
36A-LK	*Aedes aegypti*	Chiang Mai	*Leptolegnia sp.*

Note: NG = negative for fungal growth.

was isolated. Meanwhile, another genus of mosquito larvae was also tested.

Screening tests of mosquito larvae killing ability of 131 isolates of fungi were performed. Only 10 isolates (7.6% of test fungi) exhibited more than 50 % mortality when they were tested with laboratory colonized mosquito larvae. The isolate 10A-15W showed 85 % mortality when tested with *Anopheles dirus* larvae; 52.5 % when 11B-1W was tested with *Aedes aegypti* larvae; 78.7 % when 12A-6 was tested with *Aedes aegypti* larvae; 85 % and 90 % when 17A-4 was tested with *An.dirus* and *An.minimus* respectively; 95 % and 60 % when 26D-7Y was tested with *Cx. quinquefasciatus* and *Ae. aegypti* respectively; 67.5 % when 29B-5W was tested with *Cx.quinquefasciatus*; 67.5 % when 29I-2Y was tested with *An. dirus*; 77.5% when 29J-1 was tested with *Cx.quinquefasciatus* and *Ae.aegypti*; 55% when 30E-2W/P was tested with *An.dirus* and 55 % when 30E-11 was tested with *Ae.aegypti*.

After the screening test, the fungi which showed killing ability more than 50 % mortality were subsequently tested with 4 species of mosquito larvae (*Culex quinquefasciatus, Aedes aegypti, Anopheles dirus and Anopheles minimus*) in order to determine the lethal concentration (LC 50). The 29B-5W isolate was performed at concentration of 10^2 to 10^6 spores/ml. The accumulative mortality data of mosquito larvae at day 5 calculated by Reed and Muench were shown in detail (Table 3).

Table 3 Lethal concentration (LC50) of isolated fungi against 4 species of mosquito larvae.

Fungus	mosquito larvae from which fungi were isolated	Province	Lethal concentration (LC50)			
			Cx.quinque fasciatus	*Ae.aegypti*	*An.dirus*	*An.minimus*
10A-15W *Beauveria sp.*	*Culex*	Chiang Mai	2.69×10^{6}	3.09×10^{6}	1.20×10^{6}	4.42×10^{6}
11B-1W *Penicillium* like fungi	*Culex*	Chiang Mai	NC	NC	NC	NC
12A-6 *Trichoderma viride*	*Culex*	Chiang Mai	7.76×10^{5}	1.12×10^{6}	1.58×10^{6}	1.99×10^{6}
17A-4 Unidentify	*Culex*	Phitsanulok	NC	4.13×10^{6}	1.70×10^{6}	3.83×10^{5}
26D-7Y *Paecilomyces sp.*	*Culex*	Phitsanulok	NC	NC	3.53×10^{5}	3.81×10^{5}
29B-5W *Aspergillus niveus*	*Culex*	Nan	1.94×10^{4}	2.19×10^{5}	4.92×10^{4}	3.90×10^{5}
29I-2Y *Paecilomyces sp.*	*Anopheles*	Nan	NC	NC	NC	NC
29J-1 *Trichoderma sp.*	*Anopheles*	Lumpang	1.28×10^{6}	4.67×10^{6}	6.25×10^{6}	NC
30E-2W/P Unidentify	*Culex*	Chiang Rai	NC	4.21×10^{6}	3.43×10^{5}	5.75×10^{5}
30E-11 *Trichoderma sp.*	*Culex*	Chiang Rai	NC	NC	NC	2.73×10^{6}

Note: NC = LC50 could not be calculated because percent mortality at the highest fungal spore concentration testing (10^7 spores/ml) was less than 50.

Percent mortality of 11B-1W isolate tested against *Cx.quinquefasciatus*, *Ae.aegypti*, *An.dirus and An.minimus* was 7.5, 0, 7.5 and 0,respectively.

Percent mortality of 17A-4 isolate tested against *Cx.quinquefasciatus* was 2.5.

Percent mortality of 26D-7Y isolate tested against *Cx.quinquefasciatus* and *Ae.aegypti* was 5 and 0,respectively.

Percent mortality of 29I-2Y isolate tested against *Cx.quinquefasciatus*, *Ae.aegypti*, *An.dirus* and *An.minimus* was 2.5, 0, 0 and 2.5,respectively.

Percent mortality of 29J-1 isolate tested against *An.minimus* was 22.5

Percent mortality of 30E-2W/P isolate tested against *Cx.quinquefasciatus* was 7.5 and percent mortality of 30E-11 isolate tested against *Cx.quinquefasciatus*, *Ae.aegypti* and *An.dirus* was 17.5, 12.5 and 47.5,respectively.

The isolate 10A-15W (*Beauveria sp.*) and 12A-6 (*Trichoderma viride*) showed signs of fungal infection in head, thorax, abdomen, siphon, spiracular apparatus and anal papilla of tested mosquito larvae (Figure 3-10). The 10A-15W could kill larvae of *Culex quinquefasciatus*, *Aedes aegypti*, *Anopheles dirus* and *Anopheles minimus* at LC 50 = 2.69×10^6 , 3.09×10^6 , 1.20×10^6 and 4.42×10^6 spores/ml, respectively whereas the 12A-6 could do at LC50= 7.76×10^5, 1.12×10^6, 1.58×10^6 and 1.99×10^6 spores/ml , respectively Therefore , those two isolates were selected for further studies.

Two other isolates of *Trichoderma sp.* (29J-1 and 30E-11) which could infect mosquito larvae as 12A-6 but their mosquito larvae killing ability were quite less than 12A-6. Therefore, only 12A-6 isolate of *Trichoderma sp.* was selected. The 29B-5W (*Aspergillus niveus*) was also selected for further studies because it could kill mosquito larvae at low concentration; LC50 against *Cx.quinquefasciatus* was 1.94×10^4 spores/ml and LC50= 2.19×10^5, 4.92×10^4 and 3.90×10^5 spores/ml against *Ae.aegypti*, *An.dirus* and *An.minimus* respectively.

Some isolates of fungi such as 11B-1W and 29I-2Y killed more than 50 % of the mosquito larvae in screening test but the LC50 values could not be calculated because at 10^7 spores/ml they yeilded less than 50 % mortality. The 17A-4 and 26D-7Y (*Paecilomyces sp.*) killed mosquito larvae at quite high concentration of 10^5-10^6 spores/ml but fungal infection could

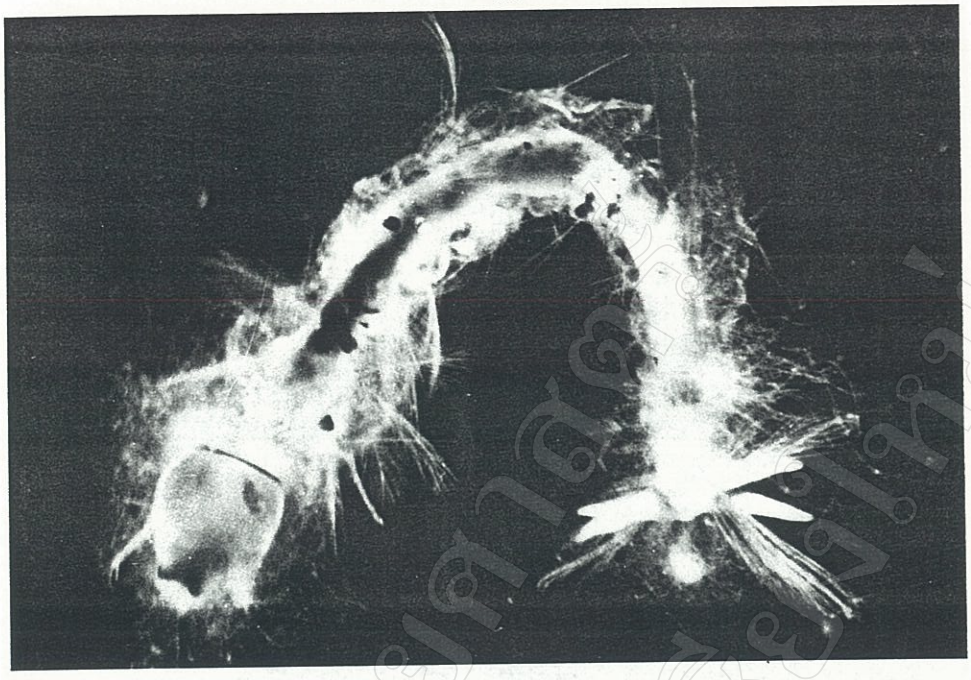

Figure 3. *Anopheles dirus* larva covered with hyphae of 10A-15W isolate of *Beauveria sp.* (Dark field microscope)

Figure 4. 12A-6 isolate of *Trichoderma viride* infection in the head of *Anopheles dirus* (x100)

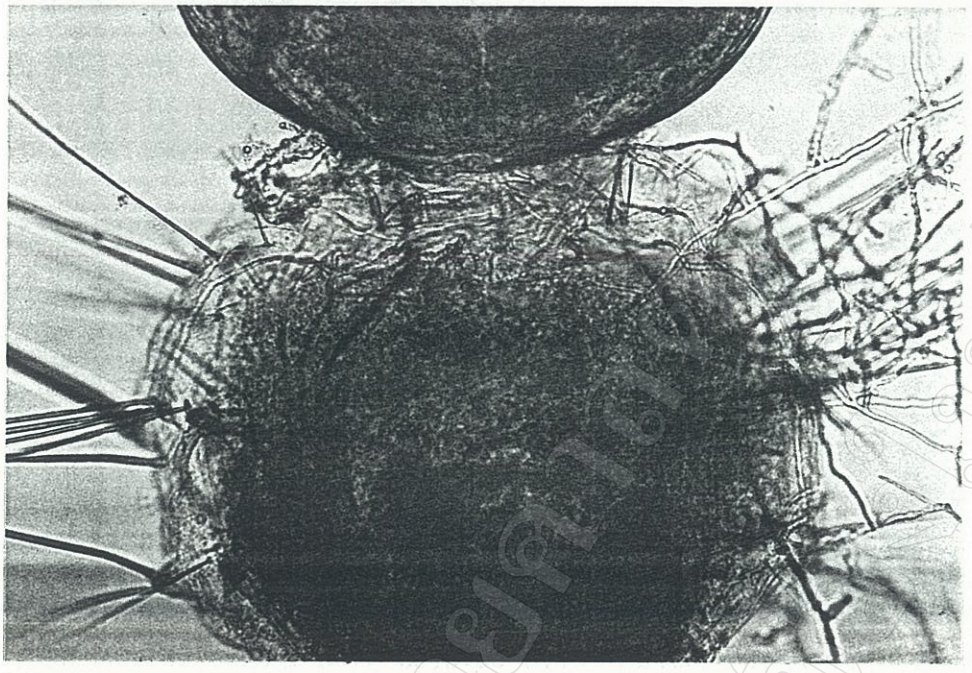

Figure 5. 12A-6 isolate of *Trichoderma viride* infection in thorax of *Culex quinquefasciatus* (x100)

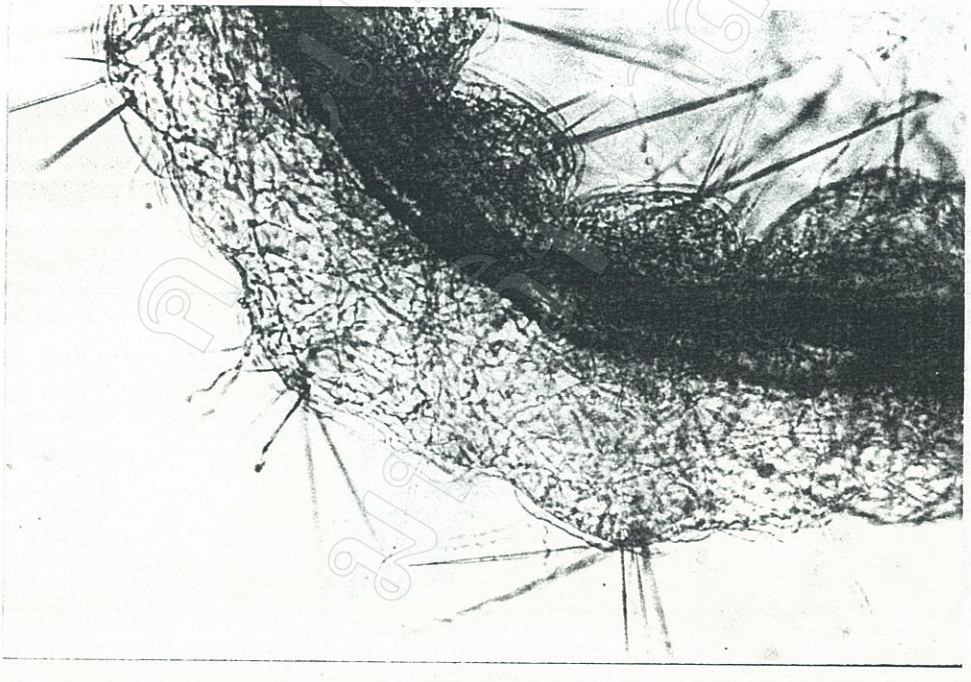

Figure 6. Abdomen of *Culex quinquefasciatus* filled with hyphae of 12A-6 isolate of *Trichoderma viride* (x100)

Figure 7. Fungal infection of 12A-6 isolate of *Trichoderma viride* in abdomen of *Aedes aegypti* (x400)

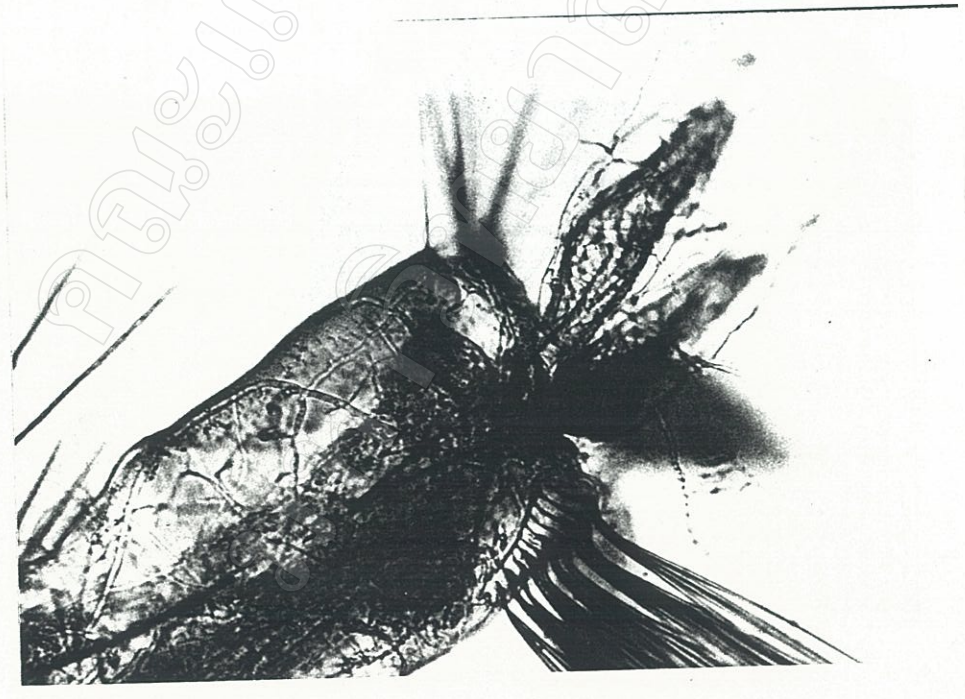

Figure 8. Fungal infection of 12A-6 isolate of *Trichoderma viride* in anal part of *Culex quinquefasciatus* larvae (x100)

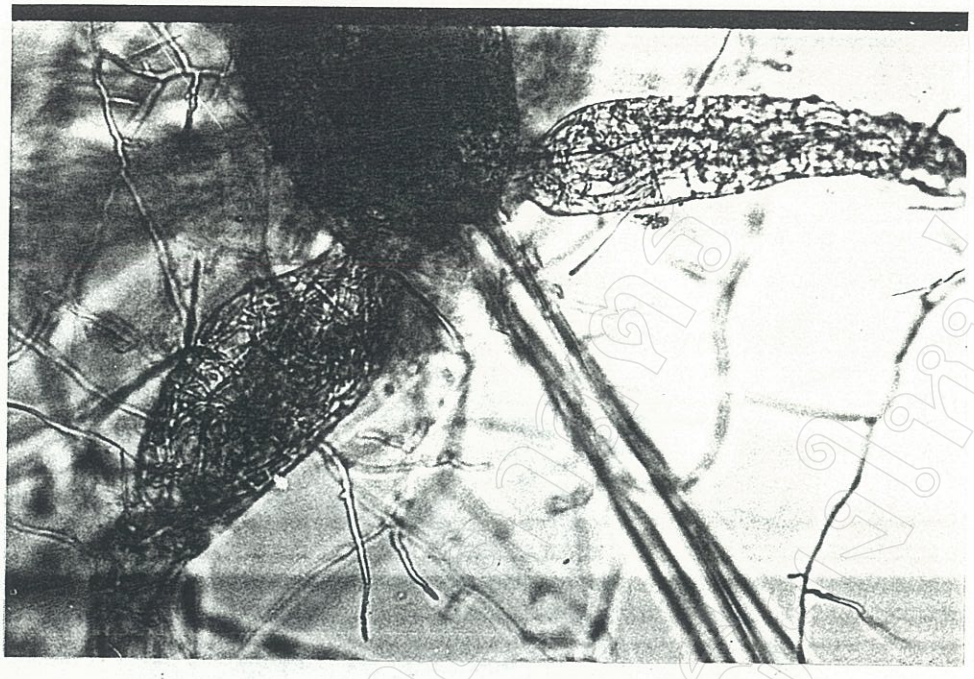

Figure 9. Fungal infection of 12A-6 isolate of *Trichoderma viride* in anal papilla of *Anopheles dirus* (x100)

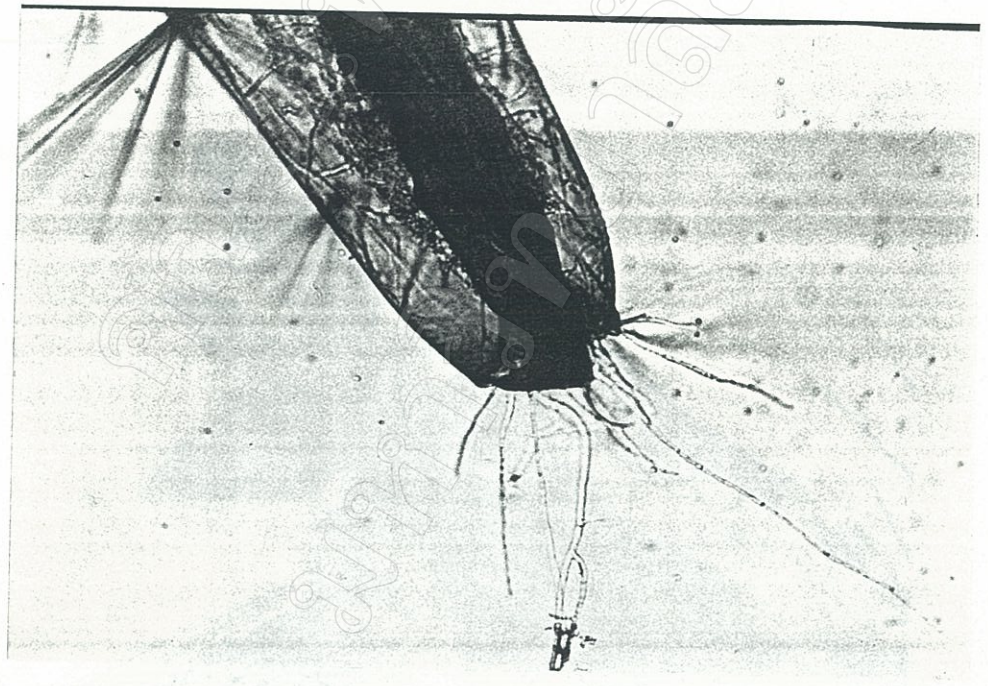

Figure 10. Fungal infection of 12A-6 isolate of *Trichoderma viride* in syphon of *Culex quinquefasciatus* (x100)

not be found in mosquito larvae. The last isolate was 30E-2W/P which killed mosquito larvae at LC50 10^5-10^6 spores/ml but it caused dark stain in water which was difficult to determine mosquitoes' mortality. Thus, the mosquito killing isolates 10A-15W, 12A-6 and 29B-5W were selected for further studies.

1. Mosquito larvae killing ability of 12A-6 isolate of *Trichoderma viride*

Mosquito larvae killing ability of 12A-6 isolate when tested against 4 species of mosquito larvae was shown in detail (Table 4). This fungus killed only small amount of mosquito larvae at low spore concentration (10^3 - 10^5 spores/ml). Mosquito larvae were killed quickly when higher concentration of fungal spored were used. At concentration of 10^6 spores/ml, 52.5% of *Cx.quinquefasciatus* were killed in 5 days whereas at 10^7 spores/ml, 77.5% of *Cx.quinquefasciatus* were killed in 1 day (Figure 11-A). The result was similar when 12A-6 isolate was tested with *Ae.aegypti* (Figure 11-B). *Anopheles dirus* and *An.minimus*, however, were killed quickly only when high spore concentration (10^7 spores/ml) was tested (Figure 11-C, 11-D). *Anopheles minimus* was less susceptible to this fungus than *An.dirus*. They were killed only 78.75% in 5 days when 10^7 spores/ml of fungus was tested. Comparing among 4 species of mosquito larvae which were tested with

Table 4 Mosquito larvae killing ability of 12A-6 isolate of *Trichoderma viride* against 4 mosquito larval species

Mosquito larvae	Concentration of fungus (spores /ml.)	% Cumulative mortality									
		1st day		2nd day		3rd day		4th day		5th day	
			average		average		average		average		average
Cx.quinquefasciatus	10^3	0		0		0		2.5		2.5	
		0	0	0	0	0	0	0	1.25	0	1.25
	10^4	0		0		0		0		0	
		0	0	0	0	0	0	2.5	1.25	5	2.5
	10^5	0		0		0		2.5		2.5	
		0	0	2.5	1.25	2.5	1.25	10	6.25	15	8.75
	10^6	10		37.5		42.5		45		55	
		17.5	13.7	30	33.7	37.5	40	47.5	46.2	50	52.5
	10^7	80		97.5		97.5		97.5		97.5	
		75	77.5	80	88.7	95	96.2	100	98.7	100	98.7
Aedes aegypti	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		0		2.5		2.5	
		0	0	0	0	0	0	0	1.25	0	1.25
	10^5	0		2.5		2.5		2.5		2.5	
		0	0	0	1.25	2.5	2.5	0	1.25	0	1.25
	10^6	22.5		37.5		42.5		45		45	
		20	21.2	30	33.7	40	41.2	40	42.5	40	42.5
	10^7	17.5		77.5		92.5		97.5		100	
		82.5	50	92.5	85	95	93.7	100	98.7	100	100
Anopheles dirus	10^3	0		0		0		0		5	
		0	0	0	0	0	0	0	0	5	5
	10^4	0		0		0		0		5	
		0	0	0	0	0	0	5	2.5	7.5	6.25
	10^5	0		0		0		0		10	
		0	0	0	0	2.5	1.25	10	5	15	12.5
	10^6	0		2.5		5		7.5		25	
		2.5	1.25	2.5	2.5	12.5	8.75	25	16.2	30	27.5
	10^7	95		100		100		100		100	
		97.5	96.2	100	100	100	100	100	100	100	100
Anopheles minimus	10^3	0		0		0		0		2.5	
		0	0	0	0	0	0	0	0	2.5	2.5
	10^4	0		0		0		0		0	
		0	0	0	0	2.5	1.25	5	2.5	2.5	1.25
	10^5	0		0		0		12.5		15	
		0	0	0	0	17.5	8.75	25	18.7	22.5	18.7
	10^6	0		0		2.5		22.5		30	
		0	0	12.5	6.25	30	16.2	40	31.2	37.5	33.7
	10^7	25		45		72.5		72.5		80	
		25	25	42.5	43.7	72.5	72.5	82.5	77.5	77.5	78.7

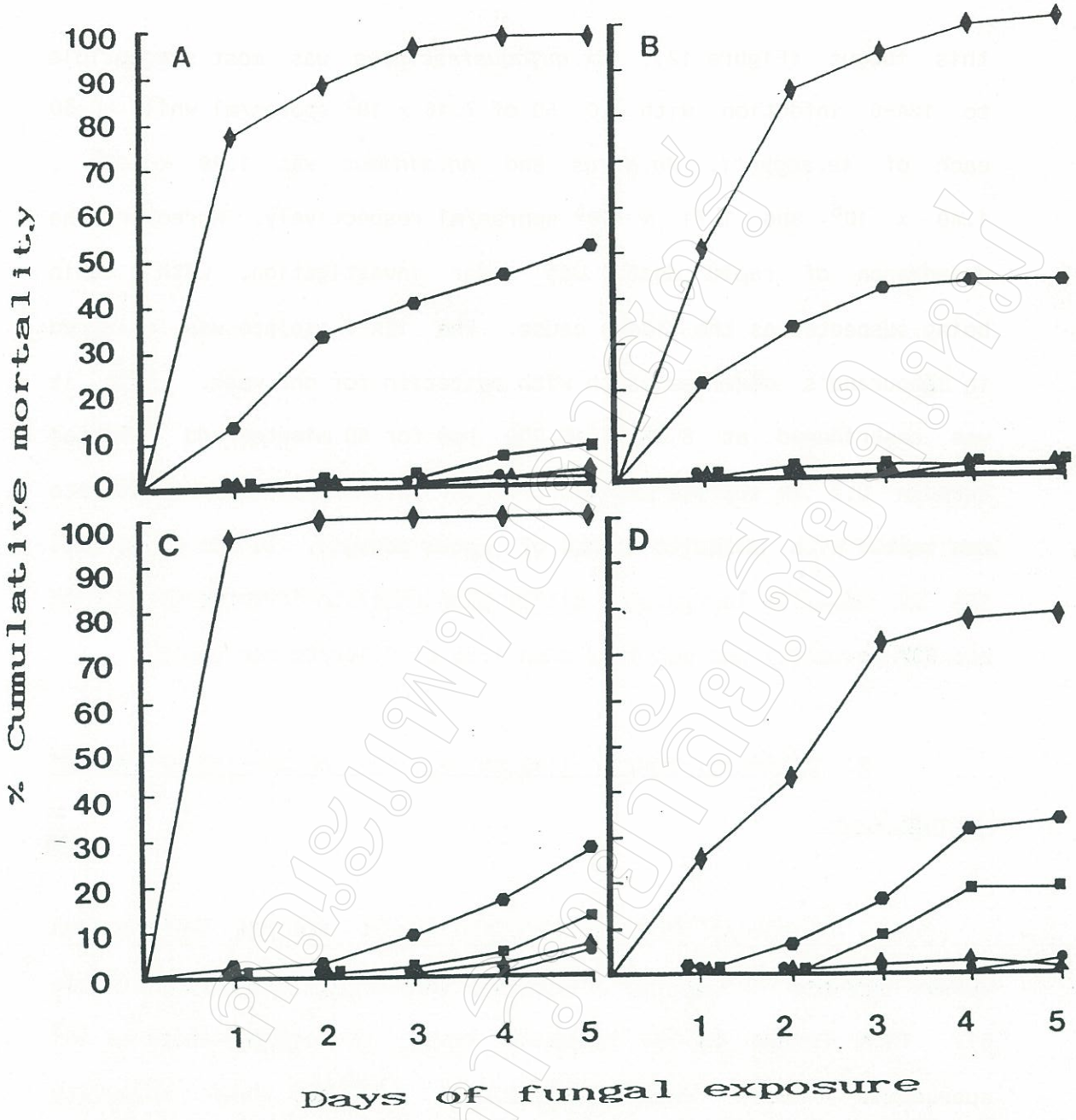

Figure 11. Cumulative mortality of mosquito larvae exposed to varying concentration of 12A-6 spores; 10^3 spores/ml (●), 10^4 spores/ml (▲), 10^5 spores/ml (■), 10^6 spores/ml (⬟) and 10^7 spores/ml (♦) at room temperature.

Panel A = *Culex quinquefasciatus*

Panel B = *Aedes aegypti*

Panel C = *Anopheles dirus*

Panel D = *Anopheles minimus*

this fungus (Figure 12), *Cx.quinquefasciatus* was most susceptible to 12A-6 infection with LC 50 of 7.16×10^5 spores/ml while LC 50 each of *Ae.aegypti*, *An.dirus* and *An.minimus* was 1.29×10^6, 1.40×10^6 and 1.71×10^6 spores/ml respectively. Moreover, the phenomenon of rapid death was under investigation, with a toxin being suspected as the likely cause. The 12A-6 isolate was cultured in Sabouraud's dextrose broth with agitation for one week. Then it was centrifuged at 8,000 - 10,000 rpm for 50 minutes and filtered through 0.2 µm sterile millipore filter before this culture filtrate was tested with mosquito larvae of *Aedes aegypti*. It was found that 35% of mosquito larvae were killed when undilute filtrate was tested and 30% mortality was obtained when 1:35 of filtrate was tested.

2. Mosquito larvae killing ability of 10A-15W isolate of *Beauveria sp.*

Mosquito larvae killing ability of 10A-15W isolate when tested against 4 species of mosquito larvae was demonstrated (Table 5). This fungus killed mosquito larvae at concentration of 10^7 spores/ml. For *Cx.quinquefasciatus* larvae, the mortality increased from day 1 to day 5 of fungal exposure reaching a maximum of 98.7% at the 5th day whereas the mortality was only 18.7% when 10^6 spores/ml of fungal spores was tested (Figure 13-A). The result was similar when this fungus was tested against *Ae.aegypti* (Figure 13-B).

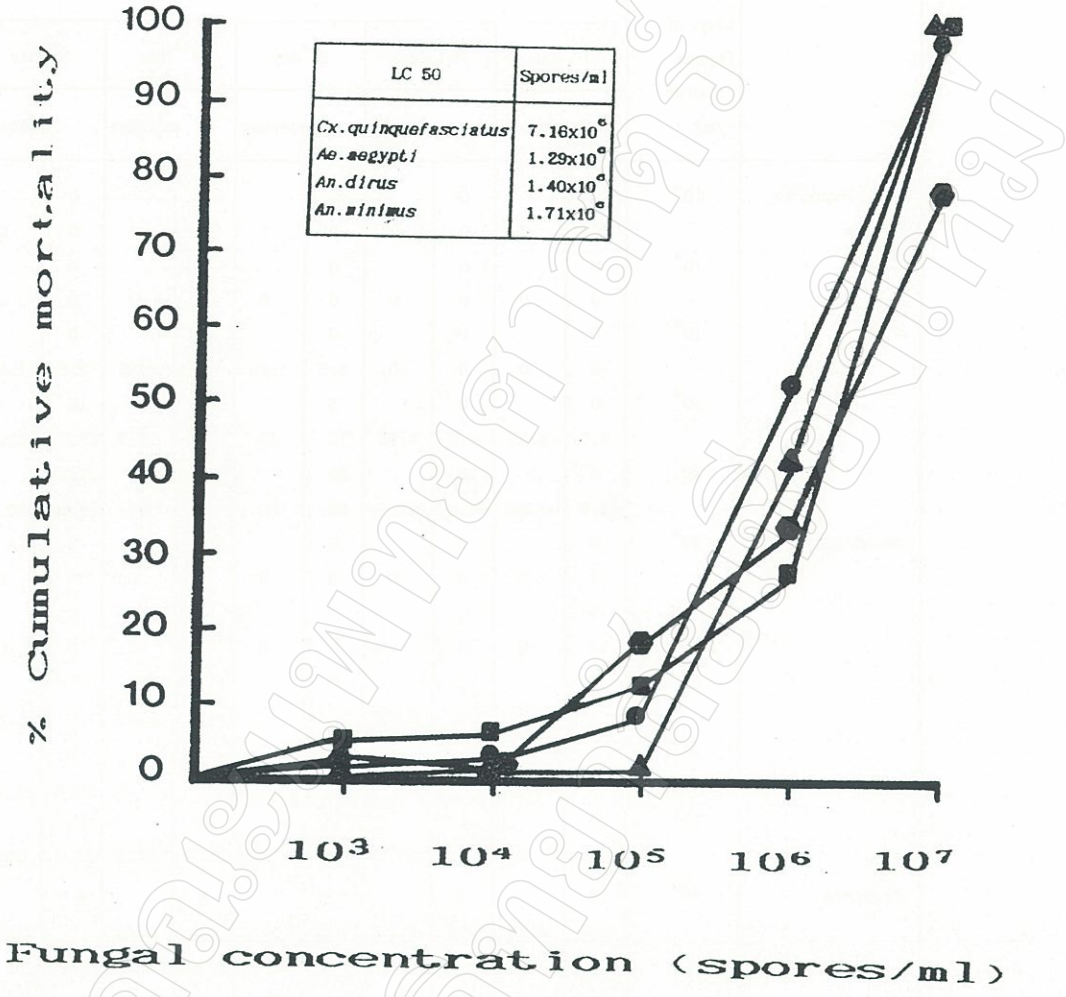

Figure 12. Percentage cumulative mortality of 4 species of mosquito larvae; *Cx. quinquefasciatus* (●), *Ae. aegypti* (▲), *An. dirus* (■) and *An. minimus* (⬢) by 12A-6 isolate of *Trichoderma viride* in various concentrations of spores. Each point represents the average percentage mortality in 5 days of duplicate tests.

Table 5 Mosquito larvae killing ability of 10A-15W isolate of *Beauveria sp.* against 4 mosquito larval species.

Mosquito larvae	Concentration of fungus (spores /ml.)	% Cumulative mortality									
		1st day		2nd day		3rd day		4th day		5th day	
			average		average		average		average		average
Cx.quinquefasciatus	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^5	0		0		0		0		0	
		0	0	0	0	2.5	1.25	2.5	1.25	2.5	1.25
	10^6	0		0		5		12.5		15	
		2.5	1.25	7.5	3.75	15	10	20	16.2	22.5	18.7
	10^7	0		20		55		95		97.5	
		12.5	6.25	42.5	31.2	65	60	95	95	100	98.7
Ae.aegypti	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^5	0		2.5		0		2.5		2.5	
		0	0	0	1.25	0	0	0	1.25	0	1.25
	10^6	0		0		0		5		5	
		0	0	2.5	1.25	7.5	3.75	10	7.5	12.5	8.75
	10^7	0		32.5		52.5		75		95	
		10	5	40	36.2	52.5	52.5	87.5	81.2	97.5	96.2
An.dirus	10^3	5		5		7.5		5		2.5	
		2.5	3.75	2.5	3.75	2.5	5	2.5	3.75	0	1.25
	10^4	0		0		2.5		7.5		7.5	
		0	0	5	2.5	10	6.25	10	8.75	10	8.75
	10^5	0		0		0		0		2.5	
		0	0	7.5	3.75	7.5	3.75	10	5	12.5	7.5
	10^6	7.5		22.5		35		47.5		52.5	
		10	8.75	17.5	20	27.5	31.2	35	41.2	47.5	50
	10^7	0		75		80		77.5		75	
		5	2.5	30	52.5	52.5	66.2	62.5	70	75	75
An. minimus	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^5	0		2.5		2.5		5		5	
		0	0	2.5	2.5	2.5	2.5	2.5	3.75	2.5	3.75
	10^6	5		12.5		17.5		17.5		20	
		2.5	3.75	5	8.75	12.5	15	17.5	17.5	22.5	21.2
	10^7	0		0		20		47.5		60	
		0	0	27.5	13.75	70	45	70	58.7	70	65

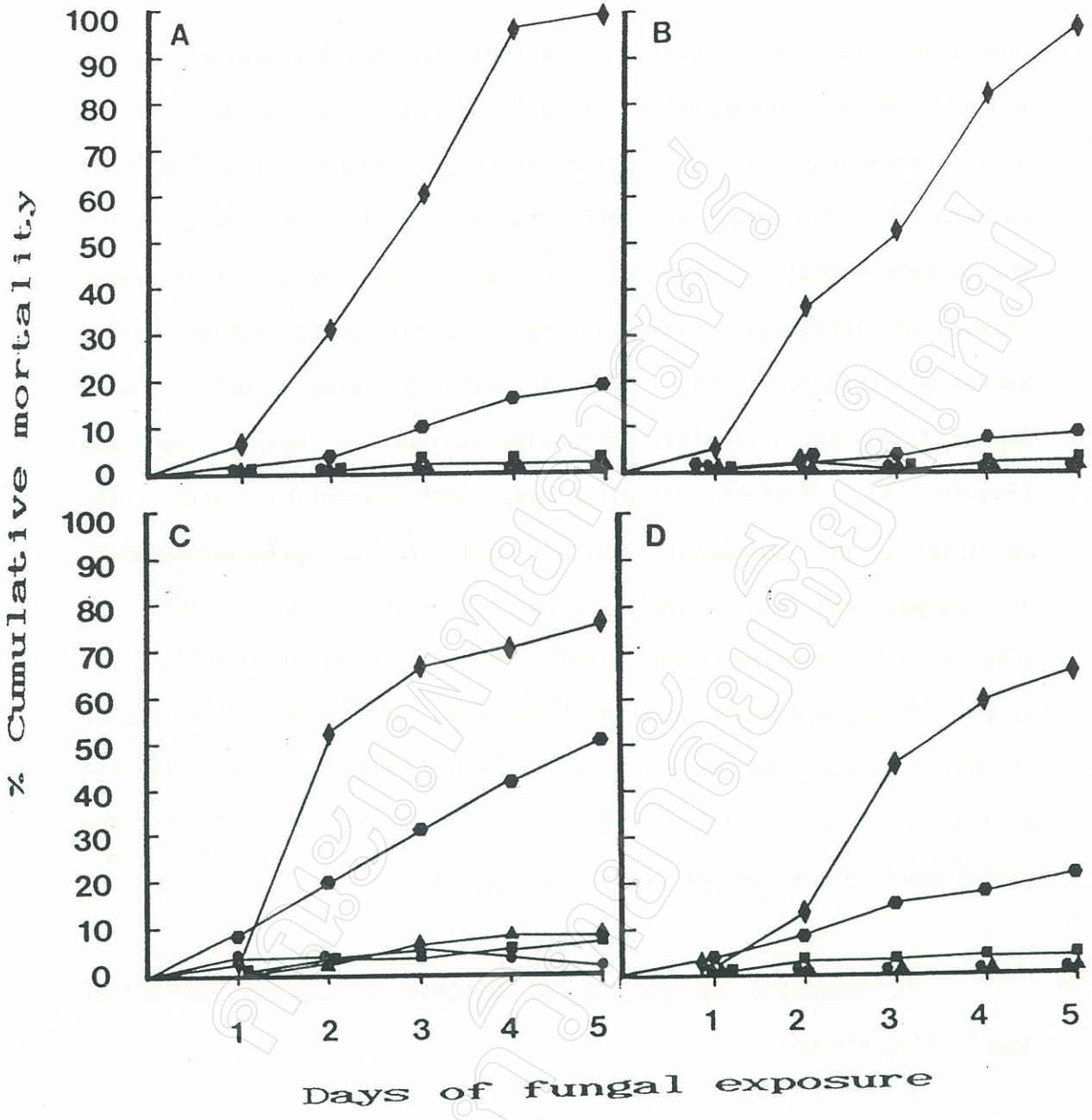

Figure 13. Cumulative mortality of mosquito larvae exposed to varying concentration of 10A-15W spores; 10^3 spores/ml (●), 10^4 spores/ml (▲), 10^5 spores/ml (■), 10^6 spores/ml (⬢) and 10^7 spores/ml (♦) at room temperature.

Panel A = *Culex quinquefasciatus*

Panel B = *Aedes aegypti*

Panel C = *Anopheles dirus*

Panel D = *Anopheles minimus*

Anopheles dirus was quite susceptible to 10A-15W isolate. It was killed 50% at concentration of 10^6 spores/ml in 5 days and 75% at concentration of 10^7 spores/ml (Figure 13-C). The mortality pattern of *An.minimus* in each day was similar to *An.dirus* but the percent mortality was less than that of *An.dirus*. In 5 days, *An.minimus* larvae were killed in 65% at fungal concentration of 10^7 spores/ml (Figure 13-D). When 4 mosquito larval species were compared, the susceptibilily to 10A-15W isolate was nearly the same (Figure 14). However, *An.dirus* was most susceptible with LC50 of 1.18×10^6 spores/ml while LC50 for *Cx. quinquefasciatus*, *Ae. aegypti* and *An. minimus* were 2.43×10^6, 2.92×10^6 and 3.96×10^6 spores/ml,respectively. Moreover, the culture filtrate of the 7 day old broth culture of 10A-15W isolate was also tested against larvae of *Ae. aegypti*. It was found that 97.5% of them were killed when the undiluted filtrate was tested and 90% of them were killed when 1:10 diluted filtrate was tested.

3. Mosquito larvae killing ability of 29B-5W isolate of *Aspergillus niveus*.

Mosquito larvae killing ability of 29B-5W isolate when tested against 4 species of mosquito larvae was shown in detail (Table 6). This fungus killed larvae of *Cx.quinquefasciatus* at low spore concentration; 38.7% of them were killed at fungal

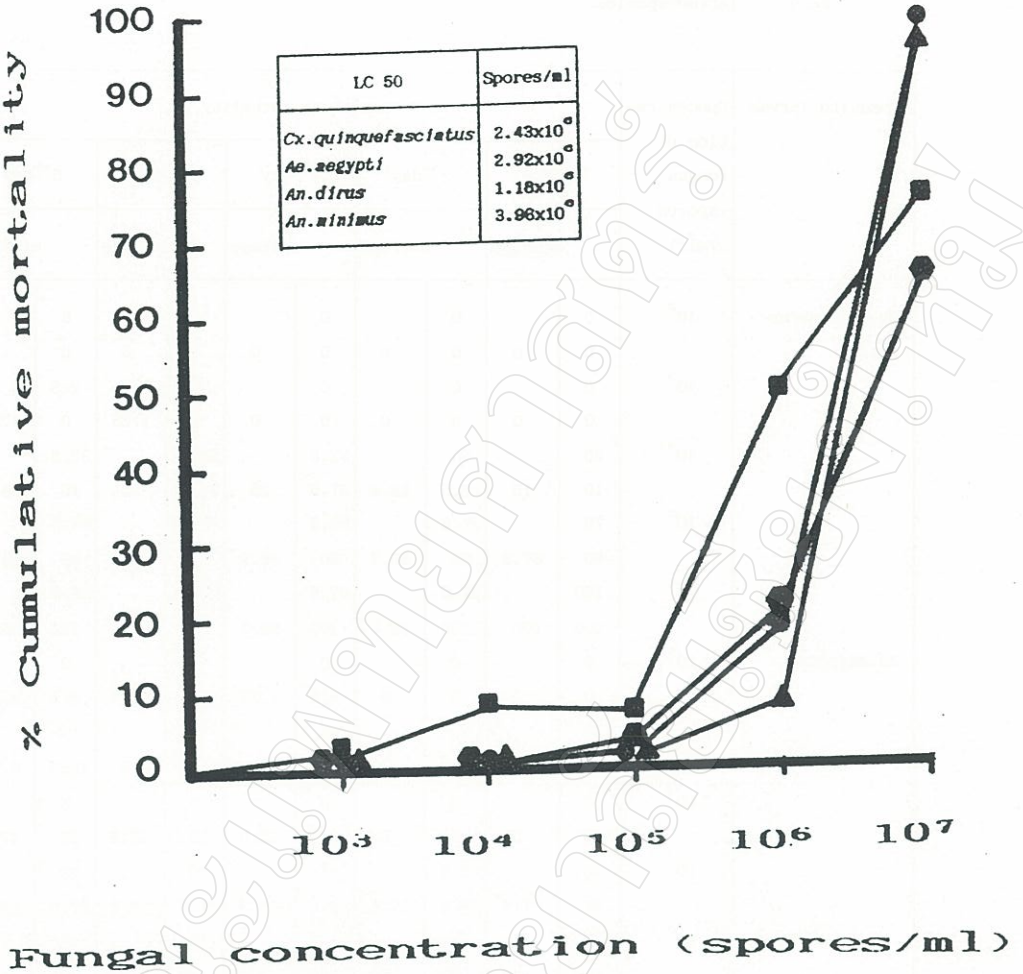

Figure 14. Percentage cumulative mortality of 4 species of mosquito larvae; *Cx. quinquefasciatus* (●), *Ae. aegypti* (▲), *An. dirus* (■) and *An. minimus* (⬢) by 10A-15W isolate of *Beauveria sp.* in various concentrations of spores. Each point represents the average percentage mortality in 5 days of duplicate tests.

Table 6 Mosquito larvae killing ability of 29B-5W isolate of *Aspergillus niveus* against 4 mosquito larvae species.

Mosquito larvae	Concentration of fungus (spores /ml.)	% Cumulative mortality									
		1st day		2nd day		3rd day		4th day		5th day	
			average		average		average		average		average
Cx.quinquefasciatus	10^2	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^3	0		0		0		2.5		2.5	
		0	0	0	0	0	0	0	1.25	0	1.25
	10^4	20		30		32.5		32.5		32.5	
		10	15	22.5	26.2	37.5	35	37.5	35	45	38.7
	10^5	75		87.5		92.5		92.5		92.5	
		60	67.5	80	83.7	80	86.2	85	88.7	85	88.7
	10^6	100		97.5		97.5		97.5		97.5	
		100	100	100	98.7	100	98.7	100	98.7	100	98.7
Ae.aegypti	10^2	0		0		0		0		0	
		0	0	0	0	2.5	1.25	2.5	1.25	2.5	1.25
	10^3	0		0		0		0		0	
		0	0	5	2.5	10	5	10	5	12.5	6.25
	10^4	0		5		5		5		5	
		0	0	5	5	20	12.5	20	12.5	30	17.5
	10^5	5		12.5		15		20		20	
		10	7.5	22.5	17.5	22.5	18.7	27.5	23.7	27.5	23.7
	10^6	100		100		100		100		100	
		100	100	100	100	100	100	100	100	100	100
An.dirus	10^2	2.5		2.5		2.5		2.5		2.5	
		0	1.25	0	1.25	0	1.25	0	1.25	0	1.25
	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		5		5		5	
		0	0	0	0	0	2.5	2.5	3.75	0	2.5
	10^5	5		25		50		67.5		67.5	
		5	5	27.5	26.2	40	45	55	61.2	55	61.2
	10^6	90		100		100		100		100	
		90	90	97.5	98.7	95	97.5	95	97.5	92.7	96.3
An.minimus	10^3	0		0		0		0		0	
		0	0	0	0	0	0	0	0	0	0
	10^4	0		0		0		0		0	
		0	0	2.5	1.25	0	0	0	0	0	0
	10^5	0		10		17.5		20		27.5	
		0	0	2.5	6.25	10	13.7	27.5	23.7	30	28.7
	10^6	2.5		25		52.5		70		77.5	
		7.5	5	20	22.5	45	48.7	70	70	72.5	75
	10^7	5		67.5		77.5		77.5		80	
		12.5	8.75	40	53.7	67.5	72.5	85	81.2	85	82.5

concentration of 10^4 spores/ml in 5 days while 67.5% were killed in one day at 10^5 spores/ml and all of them were entirely killed at fungal concentration of 10^6 spores/ml in one day (Figure 15-A). One hundred percent of *Ae. aegypti* killed at 10^6 spores/ml in one day while at lower fungal concentrations (10^2-10^5 spores/ml), small numbers of mosquito larvae were also killed (Figure 15-B). With *An. dirus*, the percent mortality in 4 days was 61.2 at fungal concentration of 10^5 spores/ml and 90% in one day at 10^6 spores/ml (Figure 15-C). *Anopheles minimus* was also tested with 29B-5W isolate. The percent mortality slightly increased from the 1st to the 5th day until it reached 75% at 10^6 spores/ml and 82.5% at 10^7 spores/ml in 5 days (Figure 15-D). The mosquito larvae killing ability of 29B-5W isolate against 4 species of mosquito larvae was compared (Figure 16). *Culex quinquefasciatus* was more susceptible to 29B-5W isolate than *An. dirus, Ae. aegypti* and *An. minimus*. The LC50 of 29B-5W isolate when being tested against *Cx. quinquefasciatus* was 1.79×10^4 spores/ml whereas the LC50 for *An. dirus, Ae. aegypti* and *An. minimus* were 6.73×10^4, 1.59×10^5 and 3.86×10^5 spores/ml, respectively.

The 29B-5W isolate was cultured in Sabouraud's dextrose broth with agitation for 7 days and the culture filtrate was also tested against mosquito larvae of *Ae. aegypti*. When the undilute filtrate was tested, 77.5% of *Ae. aegypti* were killed whereas 2.5% of them were killed when 1:10 dilution of culture filtrate was tested.

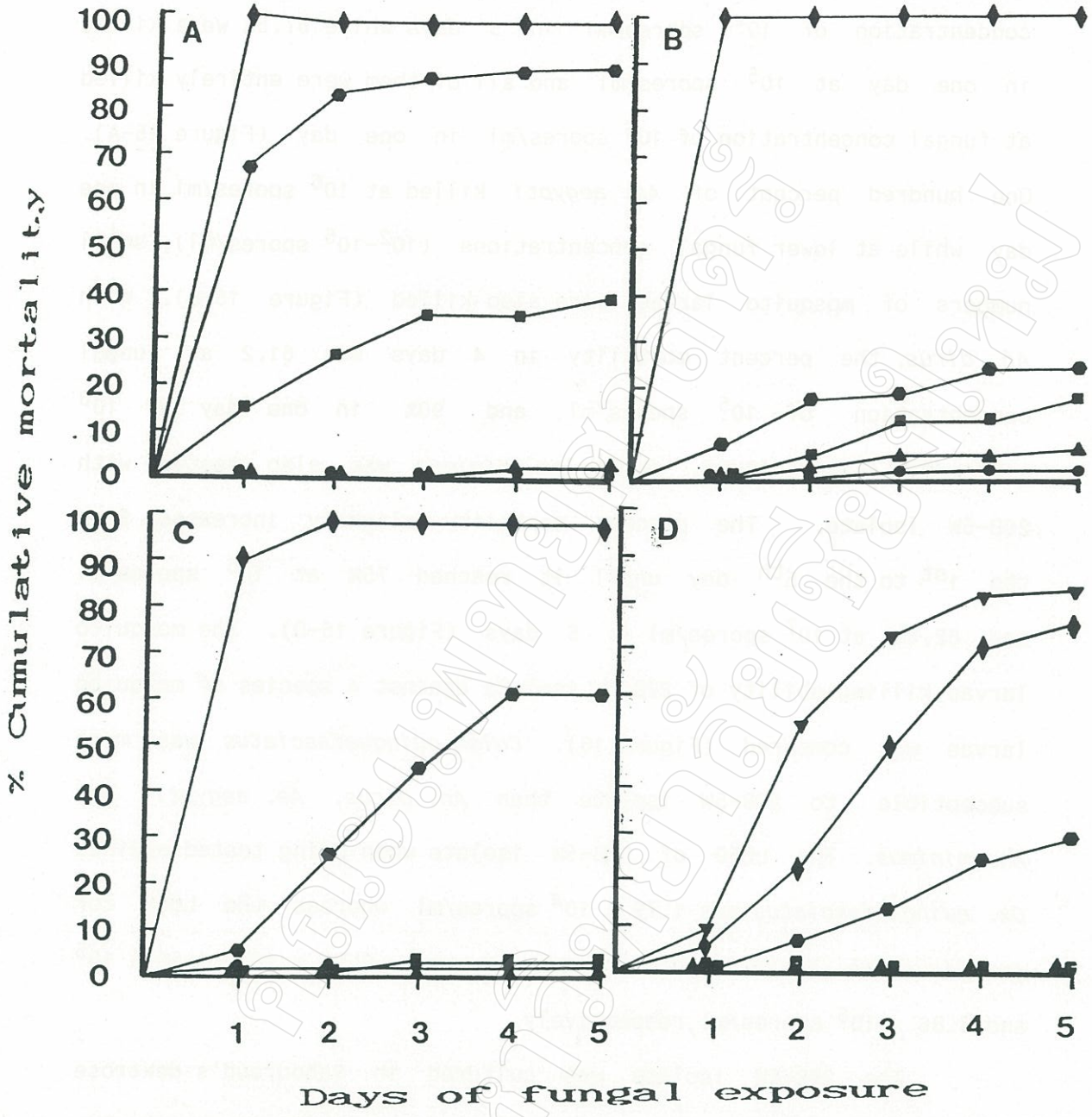

Figure 15. Cumulative mortality of mosquito larvae exposed to varying concentration of 29B-5W spores; 10^2 spores/ml (●), 10^3 spores/ml (▲), 10^4 spores/ml (■), 10^5 spores/ml (⬢), 10^6 spores/ml (♦) and 10^7 spores/ml (▼) at room temperature.

Panel A = *Culex quinquefasciatus*

Panel B = *Aedes aegypti*

Panel C = *Anopheles dirus*

Panel D = *Anopheles minimus*

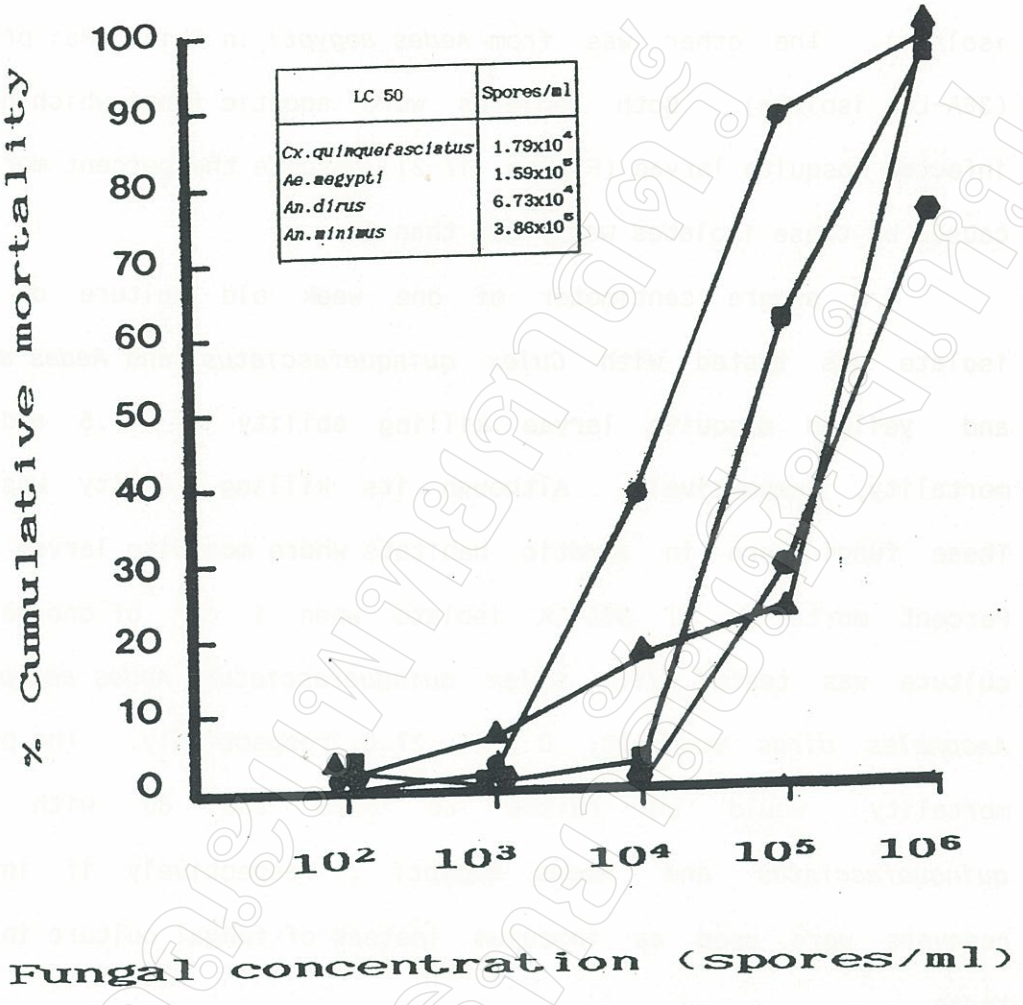

Figure 16. Percentage cumulative mortality of 4 species of mosquito larvae; *Cx. quinquefasciatus* (●), *Ae. aegypti* (▲), *An. dirus* (■) and *An. minimus* (⬣) by 29B-5W isolate of *Aspergillus niveus* in various concentrations of spores. Each point represents the average percentage mortality in 5 days of duplicate tests.

There were two interesting isolates of fungi which one was isolated from *Culex* mosquito larvae in Sukhothai province (35C-1 isolate). The other was from *Aedes aegypti* in Chiang Mai province (36A-LK isolate). Both isolates were aquatic fungi which heavily infected mosquito larvae (Figure 17-21) despite the percent mortality caused by these isolates were less than 50.

A square centimeter of one week old culture of 35C-1 isolate was tested with *Culex quinquefasciatus* and *Aedes aegypti* and yeilded mosquito larvae killing ability of 17.5 and 7.5% mortality, respectively. Although its killing ability was low. These fungi were in aquatic habitats where mosquito larvae lived. Percent mortality of 36A-LK isolate when 1 cm^2 of one week old culture was tested with *Culex quinquefasciatus, Aedes aegypti* and *Anopheles dirus* were 30, 0 and 27.5, respectively. The percent mortality would be raised to 62.5 and 80 with *Culex quinquefasciatus* and *Aedes aegypti* , respectively if infected cadavers were used as inoculum instead of fungal culture in these tests.

Being aquatic fungi, 35C-1 and 36A-LK isolates could produce zoospores.. The zoospores of 35C-1 isolate encysted after they had been released from zoosporangia but zoospores of 36A-LK isolate moved away after they had been released from zoosporangia. Hence, the former was identified as *Aphanomyces sp.*, the latter as *Leptolegnia sp.* (Akpan Eyo, 1987; Seymour, 1984; Sparrow, 1960;

Figure 17. The 35C-1 isolate of *Aphanomyces sp.* infection in head and thorax of *Culex quinquefasciatus* larvae ; zoospore encysted at the mouth part of zoosporangium (x100)

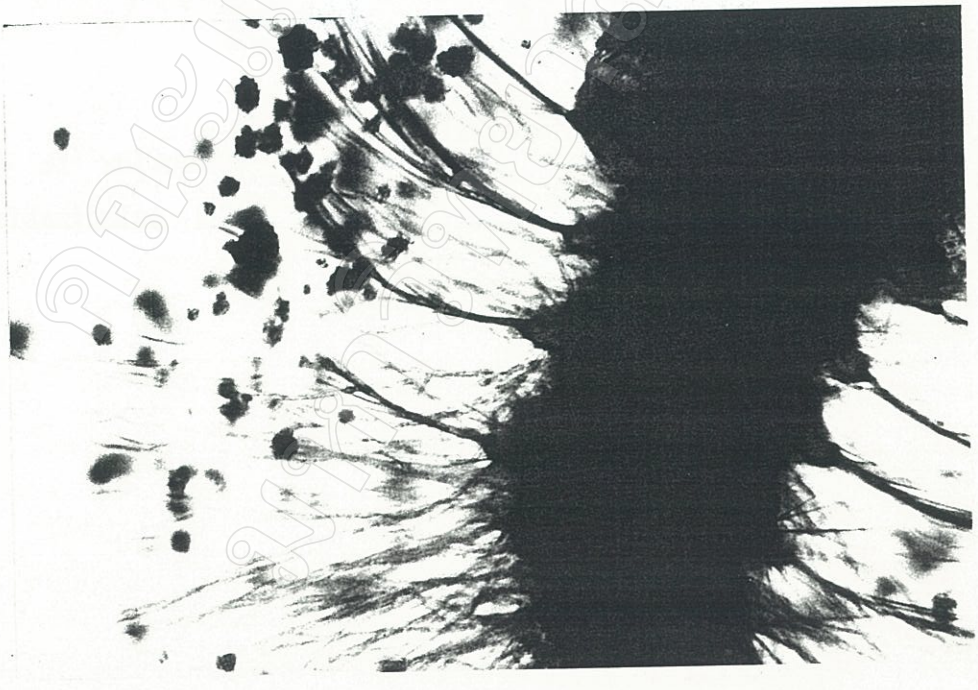

Figure 18. The 35C-1 isolate of *Aphanomyces sp.* infection in thorax and abdomen of *Culex quinquefasciatus* larvae (x100)

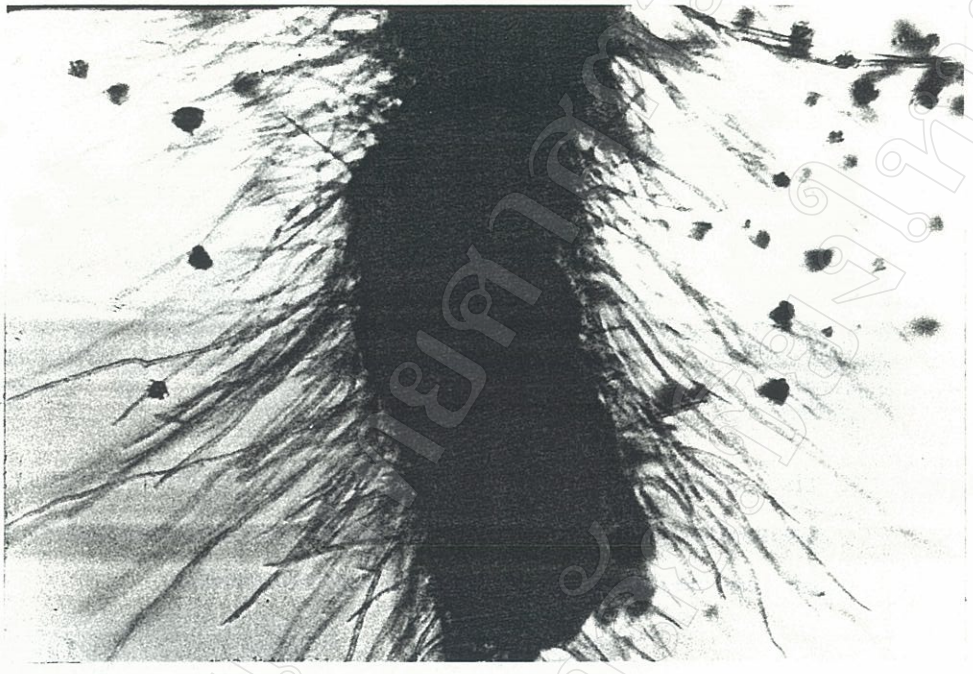

Figure 19. The 35C-1 isolate of *Aphanomyces sp.* infection in abdomen of *Culex quinquefasciatus* ; generalized melanization was usually found (x100)

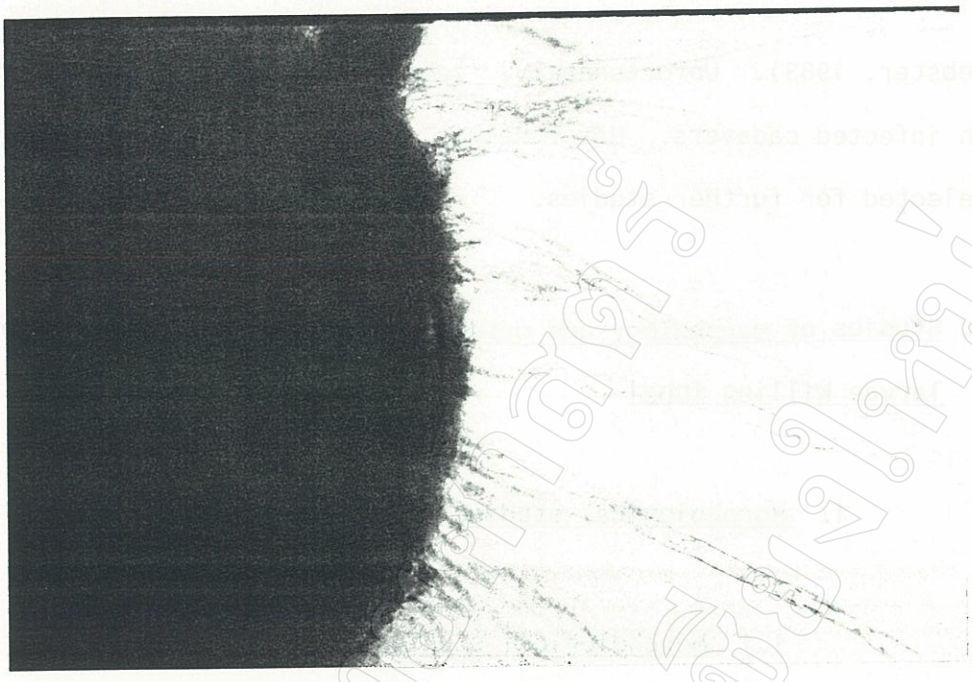

Figure 20. The 36 A-LK isolate of *Leptolegnia sp.* infection in head of *Aedes aegypti* larvae (x100)

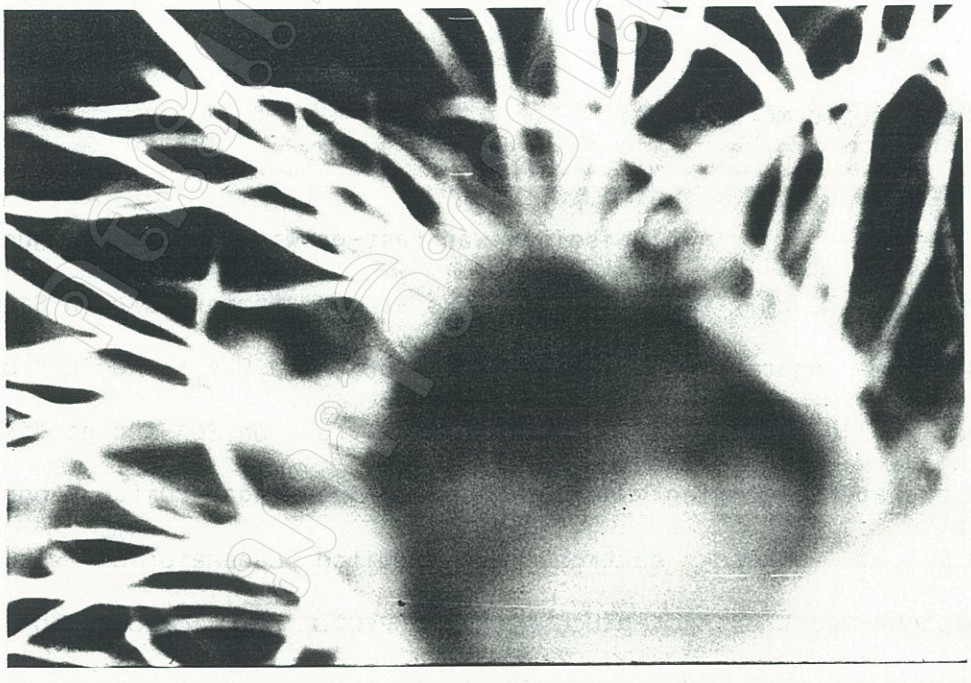

Figure 21. The 36A-LK isolate of *Leptolegnia sp.* infection in head of *Aedes aegypti* larvae (Dark field microscope x40)

Webster, 1983). Unfortunately, zoospore production was found only in infected cadavers. Nevertheless, there two aquatic fungi were also selected for further studies.

C. Studies of morphology and optimal conditions for growth of mosquito larvae killing fungi

1. Morphological studies

1.1 Morphological studies of 12 A-6 isolate

12 A-6 isolate was identified as *Trichoderma viride* Pers. ex Gray (1821) (Domsch *et al.*, 1986)

Macroscopic morphology:

This isolate was fast-growing hyaline fungus. Colony reached 7 cm. diam. in 4 days at room temperature on Sabouraud's dextrose agar (SDA) (Difco Laboratories, U.S.A.). Colour of colony was yellowish, bright, green to dark green on SDA and at first white but later becoming dark green on cornmeal agar (Difco Laboratories, U.S.A.), potato dextrose agar (Difco Laboratories, U.S.A) and peptone-yeast extract-glucose agar (PYG). Their compact conidiophore tufts were appeared with fluffy to floccose in textures (Figure 22).

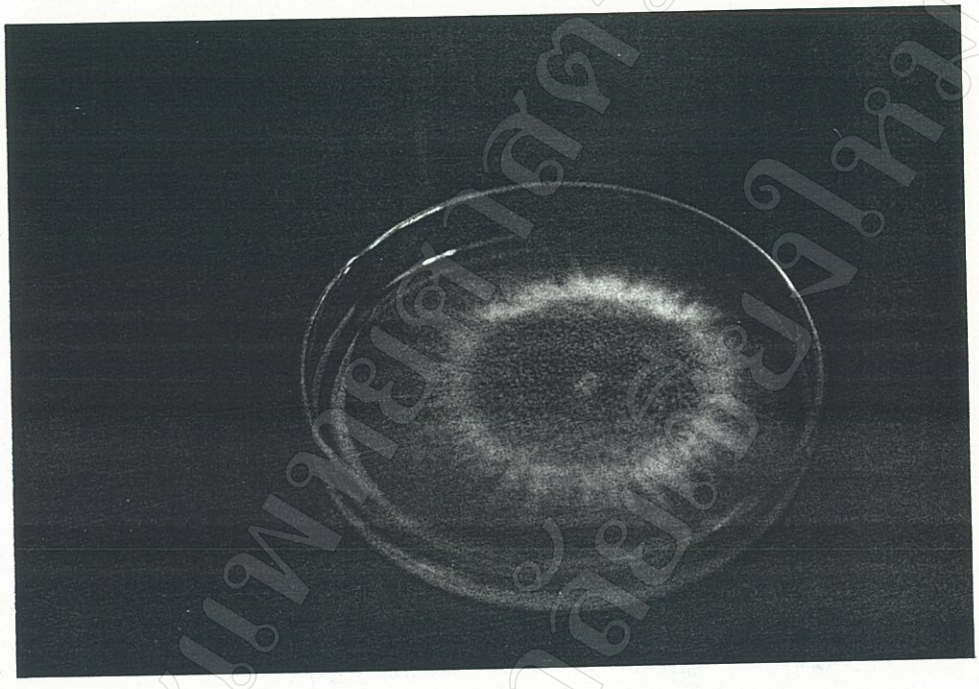

Figure 22. Macroscopic appearance of 12A-6 isolate of *Trichoderma viride* colony on SDA for 3 day culture at room temperature.

Microscopic morphology:

Hyphae were hyaline (3-8 µm wide) and septate. Conidiophores typically pyramidally branced, short branches occured near the tip and longer ones with repeated branching in the lower part. Phialides arranged in divergent groups of 2-4, slender and irregularly bent. Conidia were usually green and almost globose, 3.6-4.5 µm diam. (or to 4.8 µm long). Hyaline chlamydospore were usually present in the mycelium of older cultures (Figure 23).

Figure 23. Microscopic appearance of 12A-6 isolate of *Trichoderma viride* (x400)

1.2 Morphological studies of 10A-15W isolate

10A-15W isolate was identified as *Beauveria sp.* Vuill. (1912) (Domsch *et al.*, 1986; Emmons *et al.*, 1977)

Macroscopic morphology:

Colony grew slowly, mostly not exceeding 3 cm. diam. in ten days at room temperature on SDA , wooly, floccose, often appearing powdery due to the abundant conidia, at first white but later often becoming pale yellow (Figure 24).

Microscopic morphology:

Hyphae were hyaline and narrow (1-3 ʌm wide). Conidiogenous apparatus formed dense clusters of flask - shaped basal cells which tapered to slender spore - bearing portion and a zig-zag shaped. After prolonged periods in culture, the conidiogenous cells tended to be more slender and less densely clustered. Conidia was small (2-3 ʌm. diam.), hyaline and round to oval in shape (Figure 25).

Figure 24. Macroscopic appearance of 10A-15W isolate of *Beauveria sp.* colony on SDA after 2 week culture at room temperature.

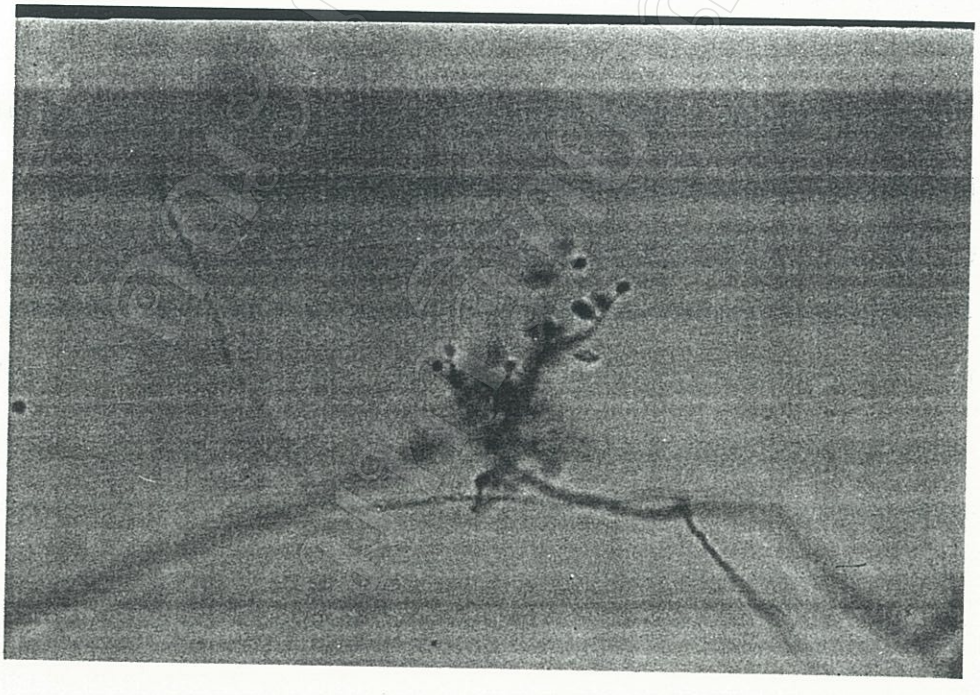

Figure 25. Microscopic appearance of 10A-15W isolate of *Beauveria sp.* (x400)

1.3. Morphological studies of 29B-5W isolate

29B-5W isolate was identified as *Aspergillus niveus* Blochwitz (1929) telomorph: *Emericella nivea* Wiley & Simmons (1973) (Domsch *et al.*, 1986)

Macroscopic morphology:

It was fast - growing fungus. Colony reached 8.9 cm. diam. in 7 days on SDA, white at first but becoming pale lemon to cream with age. The exudate was pale to brown (Figure 26).

Microscopic morphology:

This fungus was characterized as *Aspergillus niveus* by the presence of erect conidiophores which terminated in a small conidial heads (vesicle) which white at first but becoming cream with age, radiating to lossely columnar; conidiophore wall hyaline, metulae covering the upper three - quarters of vesicle. Conidia were globose, smooth - walled, 2.4 -3.2 μm. diam. Ascospores appeared faintly yellow, lenticular with very low equatorial crests, 4.0 - 5.6 x 3.2 - 4.8 μm (Figure 27).

Figure 26. Macroscopic appearance of 29B-5W isolate of *Aspergillus niveus* colony on CMDP after a week culture at room temperature.

Figure 27. Microscopic appearance of 29B-5W isolate of *Aspergillus niveus* (x400)

1.4. Morphological studies of 35C-1 isolate

35C-1 isolate was identified as *Aphanomyces sp.* de Bary (1850) (Sparrow, 1960; Webster, 1983)

Macroscopic morphology:

Colony was white to cream, fluffy and grew rapidly It reached 8.4 cm. diam. in three days at room temperature on SDA and produced an unpleasant odour (Figure 28).

Microscopic morphology:

Hyphae were 4-10 µm. wide, delicate, hyaline and non-septate which formed narrow sporangia containing a single row of zoospores; after an apical rupture of the sporangium, protoplasts were cleaved out which move to the mouth of the sporangium and encysted almost immediately after emerging and formed a loose mass of encysted spores near the mouth of the sporangium until they emerged for a second swarming phase. This occured in the manner typical of this genus (Figure 29).

Figure 28. Macroscopic appearance of 35C-1 isolate of *Aphanomyces sp.* colony on SDA for 2 day culture at room temperature.

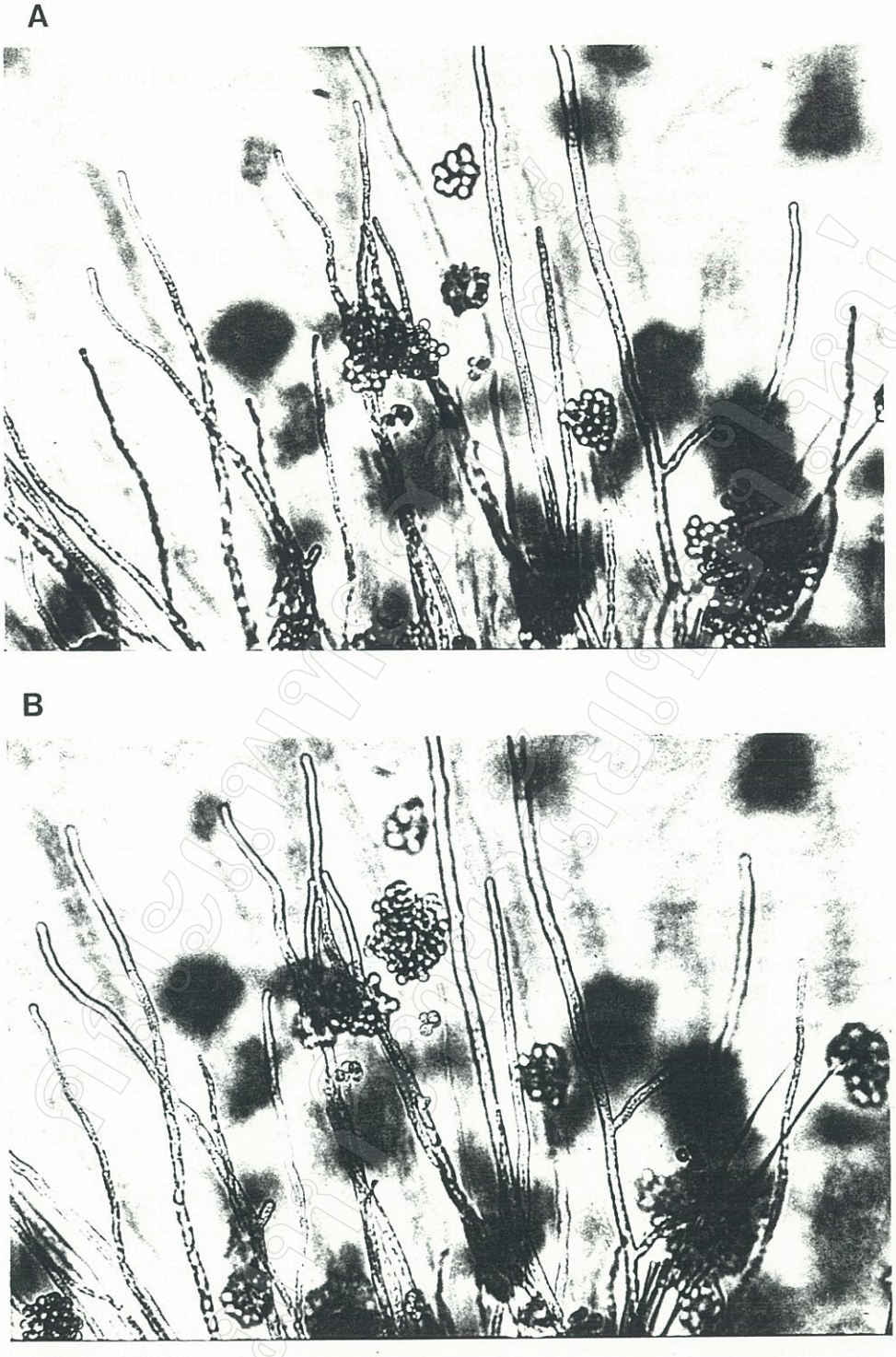

Figure 29. Microscopic appearance of 35C-1 isolate of *Aphanomyces sp.*
(A). Zoospore discharged from a pore at the tip of sporangium
(B). Zoospore encysted at the mouth of sporangium

1.5. Morphological studies of 36A-LK isolate

36A-LK isolate was identified as *Leptolegnia sp.* de Bary (1888) (Akpan Eyo, 1987; Seymour, 1984; Sparrow, 1960)

Macroscopic morphology:

This fungus was aquatic and fast-growing. Colony reached 7.8 cm. diam. in 2 days at room temperature on SDA. Colony was white fluffy to cottony in textures (Figure 30).

Microscopic morphology:

Hyphae were non-septate, long and delicate, 10-18 μm in diameter. Sporangia were long, apical, cylindric, the same size as the hyphae, and rarely branched. Spores, formed in a single row within the sporangia (zoosporangia) were elongated on discharge, then formed an oval shape and swam immediately after discharging from a pore at the tip of the sporangia. The motile primary zoospores swam for a period of time before encysting.

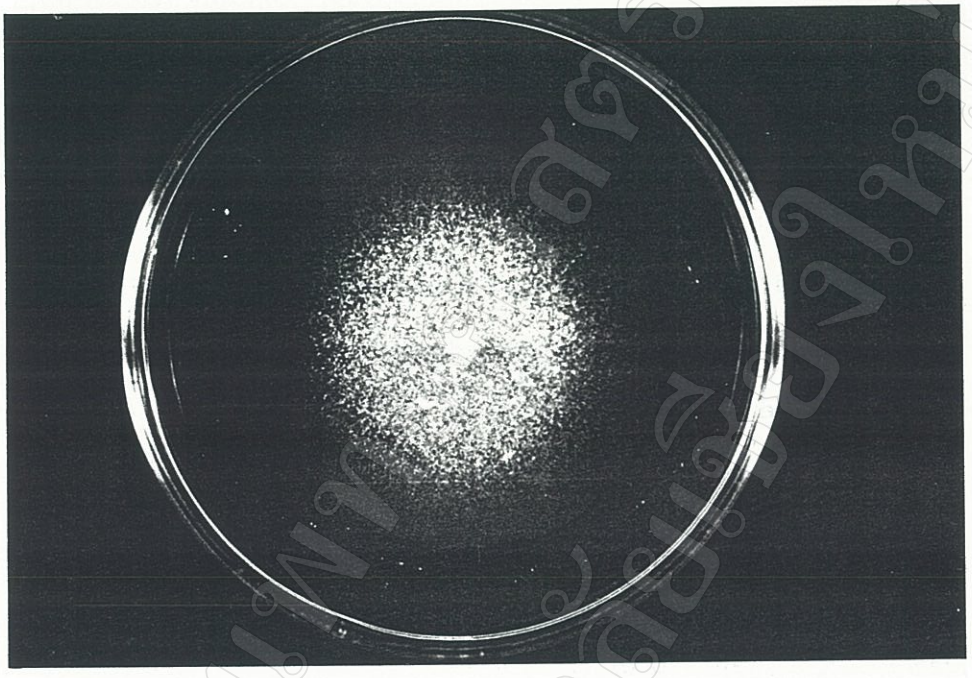

Figure 30. Macroscopic appearance of 36 A-LK isolate of *Leptolegnia sp.* colony on SDA for 2 day culture at room temperature.

Fungal growth and spore production of mosquito larvae killing fungi on different media.

Fungal growth and spore production were determined when mosquito larvae killing fungi were cultured on different media; peptone-yeast extract-glucose (PYG), potato dextrose agar (PDA) (Difco Laboratories, U.S.A.). Sabouraud's dextrose agar (SDA) (Difco Laboratories, U.S.A) and cornmeal-dextrose-peptone agar (CMDP). This experiment was performed at room temperature.

The fungal growth of 12A-6 isolate (*Trichoderma viride*) was determined by measuring colonial diameters after they had been allowed to grow on various media as described previously (Table 7, Figure 31). This isolate grew fast when it was cultured on CMDP and PDA. It grew covering petri dish in 4 days whilst it took 5 days and 6 days to cover petri dish of SDA and PYG, respectively. The growth on CMDP was significantly higher than on PYG ($P<0.05$). According to dry weight of 2 day cultures, it was found that dry weight was highest (41.8 mg) when cultured on SDA ($p<0.05$) (Table 8). Spore enumeration of this isolate was also determined. Spore production was highest when being cultured on CMDP (Table 9, Figure 32).

Growth of 10A-15W isolate of *Beauveria sp.* on various media was also determined. It was found that the fungal growth was nearly the same according to their colonial diameters (Table 10,

Table 7 Growth of 12A-6 isolate of *Trichoderma viride* on different media at room temperature determined by diameter of colonies.

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
1	1.20		2.70		1.60		2.50	
	1.20	1.20	2.70	2.70	1.60	1.60	2.50	2.50
	1.20		2.70		1.60		2.50	
2	2.50		5.00		3.30		5.00	
	2.50	2.50	6.00	5.50	3.30	3.36	5.00	5.10
	2.50		5.50		3.50		5.30	
3	4.00		7.00		6.00		7.70	
	4.50	4.16	8.50	7.73	5.50	5.66	8.00	7.80
	4.00		7.70		5.50		7.70	
4	6.00		9.00		7.20		9.00	
	6.00	6.06	9.00	9.00	7.00	7.13	9.00	9.00
	6.20		9.00		7.20		9.00	
5	9.00		9.00		9.00		9.00	
	8.50	8.83	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
6	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

Table 7 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
7	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
8	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
9	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
10	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
11	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
12	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

Table 7 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
13	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
14	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

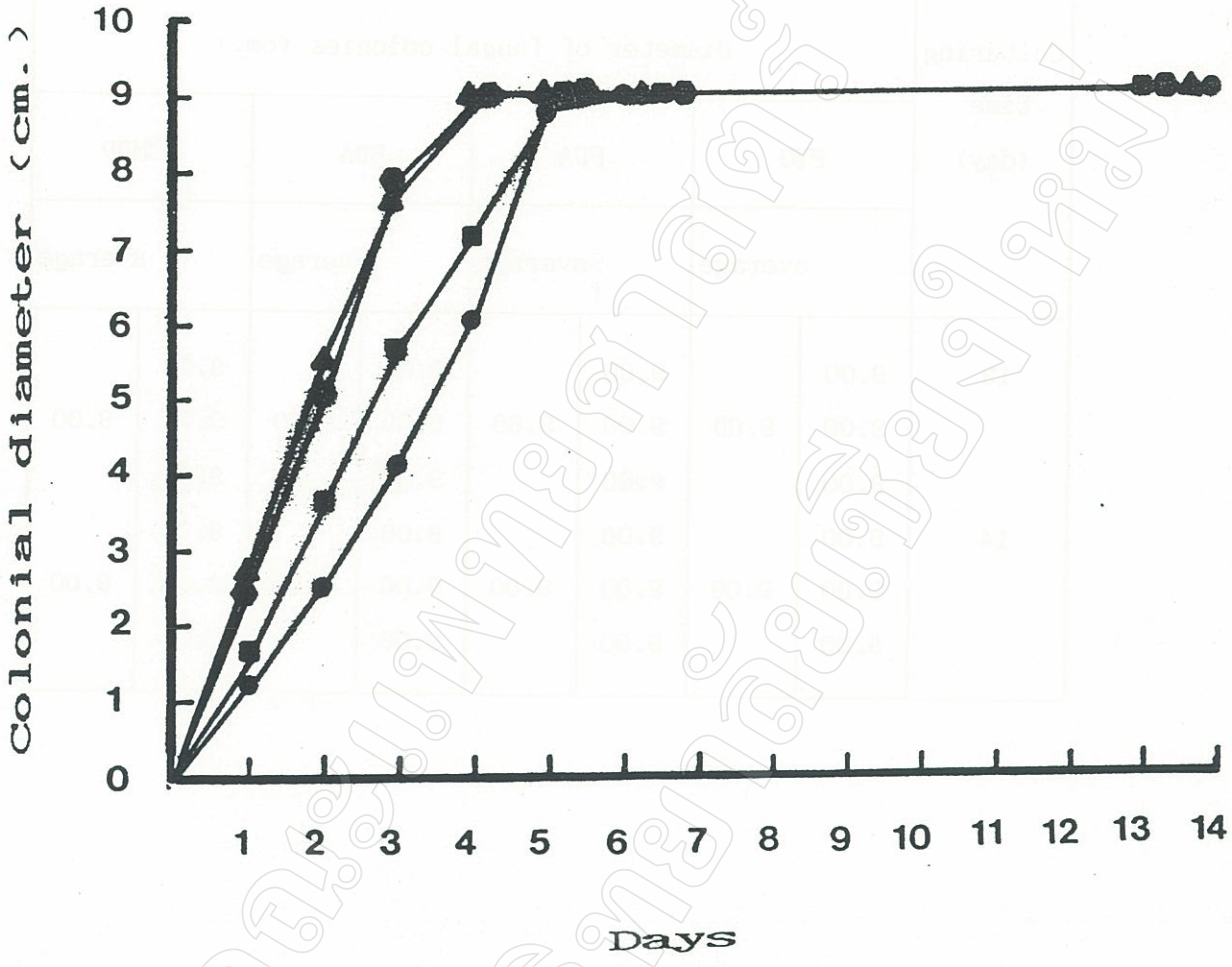

Figure 31. Growth of 12A-6 isolate of *Trichoderma viride* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at room temperature determined by colonial diameters. Results represent the average of triplicate cultures.

Table 8 Growth of 12A-6 isolate of *Trichoderma viride* on different media at room temperature determined by dry weight of 2 days old cultures.

Fungus	Dry weight (milligrams)			
	PYG[a]	PDA[b]	SDA[c]	CMDP[d]
12A-6 isolate of *Trichoderma viride*	4.4 4.2	32.5 32.4	43.4 40.1	22.5 23.6
average	4.3	32.4	41.8	23.0

Note : a. PYG = Peptone - yeast extract - glucose agar

b. PDA = Potato dextrose agar

c. SDA = Sabouraud's dextrose agar

d. CMDP = Cornmeal - dextrose - peptone agar

Table 9 Spore production of 12A-6 isolate of *Trichoderma viride* cultured on different media at room temperature.

Culturing time (day)	Spore enumeration (Spores/ml)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
3	3.54×10^{6}		4.92×10^{6}		4.27×10^{5}		1.20×10^{7}	
	4.92×10^{6}	4.23×10^{6}	6.98×10^{6}	5.95×10^{6}	6.07×10^{5}	5.17×10^{5}	1.64×10^{7}	1.42×10^{7}
5	7.05×10^{6}		2.69×10^{7}		3.42×10^{6}		7.57×10^{7}	
	8.95×10^{6}	8.00×10^{6}	3.31×10^{7}	3.00×10^{7}	2.58×10^{6}	3.00×10^{6}	6.43×10^{7}	7.00×10^{7}
7	3.14×10^{7}		1.93×10^{8}		1.87×10^{7}		4.28×10^{8}	
	4.85×10^{7}	3.99×10^{7}	1.07×10^{8}	1.50×10^{8}	2.75×10^{7}	2.31×10^{7}	3.14×10^{8}	3.71×10^{8}
14	1.03×10^{7}		1.02×10^{8}		2.02×10^{6}		1.94×10^{8}	
	1.22×10^{7}	1.12×10^{7}	3.32×10^{8}	2.17×10^{8}	2.83×10^{6}	2.42×10^{6}	1.65×10^{8}	1.79×10^{8}

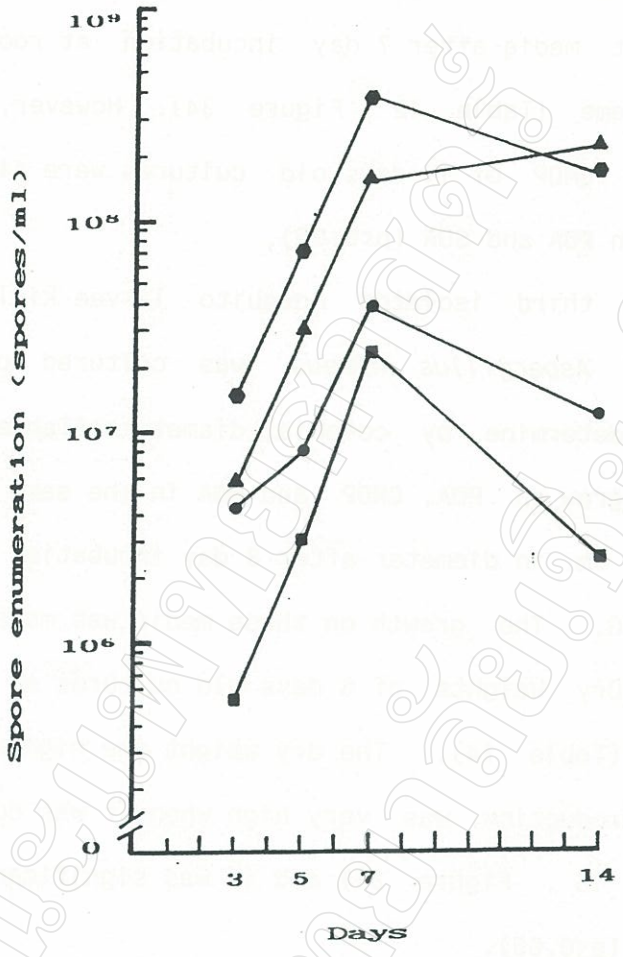

Figure 32. Spore production of 12A-6 isolate of *Trichoderma viride* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at different incubation time. Results represent the average of duplicate cultures.

Figure 33). On SDA, the fungal growth rate was slowest ($p<0.01$) and the dry weight was highest ($p<0.05$) (Table 11). Spore productions on different media after 7 day incubation at room temperature were nearly the same (Table 12 , Figure 34). However, spore productions on PYG and CMDP of 14 days old cultures were significantly higher than those on PDA and SDA ($p<0.05$).

The third isolated mosquito larvae killing fungus, 29B-5W isolate of *Aspergillus niveus*, was cultured on various media. Growth was determine by colonial diameters (Table 13 , Figure 35). This fungus grew on PDA, CMDP and SDA in the same rate (Figure 35), reaching 9 cm. in diameter after 8 day incubatian whereas it took 11 days on PYG. The growth on these media was more rapid than on PYG ($p<0.01$). Dry weights of 6 days old cultures on various media were determined (Table 14). The dry weight was highest on SDA ($p<0.05$). Ascospore production was very high when it was cultured on CMDP and PDA (Table 15 , Figure 36) and it was significantly higher than on SDA and PYG ($p<0.05$).

Growth of 35C-1 isolate of *Aphanomyces sp.* on different media was determined by measuring the diameter of fungal colonies (Table 16, Figure 37). This fungus was fast-growing, and the colony cultured on PDA reached 9 cm. in diameter within 3 days. However, the fungal growth on different media was not significantly different ($p>0.05$). The dry weight of 2 days old cultures was highest (73.2 mg) on SDA ($p<0.05$) and less on PDA (42.8 mg) and on CMDP (41.6 mg).

Table 10 Growth of 10A-15W isolate of *Beauveria sp.* on different media at room temperature determined by diameter of colonies.

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
1	0.20		0.20		0.20		0.20	
	0.20	0.20	0.20	0.20	0.20	0.20	0.20	2.20
	0.20		0.20		0.20		0.20	
2	0.50		0.50		0.50		0.55	
	0.50	0.50	0.50	0.51	0.60	0.58	0.60	0.51
	0.50		0.55		0.65		0.40	
3	0.90		0.75		1.05		1.00	
	1.00	0.95	0.90	0.88	0.90	0.95	1.10	1.00
	0.95		1.00		0.90		0.90	
4	1.30		1.25		1.25		1.35	
	1.25	1.30	1.25	1.30	1.25	1.23	1.60	1.41
	1.35		1.40		1.20		1.30	
5	1.65		1.60		1.45		1.65	
	1.70	1.61	1.65	1.68	1.45	1.43	1.95	1.71
	1.50		1.80		1.40		1.55	
6	1.95		1.95		1.60		2.25	
	1.95	1.86	2.00	2.03	1.60	1.63	2.25	2.05
	1.70		2.15		1.70		1.65	

Table 10 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
7	2.40		2.70		2.05		2.70	
	2.40	2.50	2.60	2.71	2.00	2.01	2.70	2.51
	2.70		2.85		2.00		2.15	
8	2.80		2.90		2.15		2.90	
	2.75	2.90	2.90	3.00	2.40	2.26	3.30	2.90
	3.15		3.20		2.25		2.50	
9	3.20		3.30		2.40		3.15	
	3.20	3.46	3.15	3.35	2.55	2.43	3.65	3.25
	4.00		3.60		2.35		2.95	
10	3.60		3.80		2.50		3.50	
	3.70	3.70	3.90	3.90	2.55	2.50	3.40	3.40
	3.80		4.00		2.45		3.30	
11	4.10		4.30		2.80		3.80	
	4.10	4.10	4.40	4.40	2.80	2.80	3.80	3.80
	4.10		4.50		2.80		3.80	
12	4.50		4.80		3.00		4.30	
	4.50	4.50	4.90	4.90	3.00	3.00	4.20	4.30
	4.50		5.00		3.00		4.40	

Table 10 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
13	4.80		5.30		3.40		4.80	
	4.90	4.90	5.40	5.40	3.30	3.30	4.80	4.80
	5.00		5.50		3.20		4.80	
14	5.20		5.70		3.75		5.10	
	5.40	5.36	6.00	5.93	4.35	3.73	5.20	5.26
	5.50		6.10		3.10		5.50	

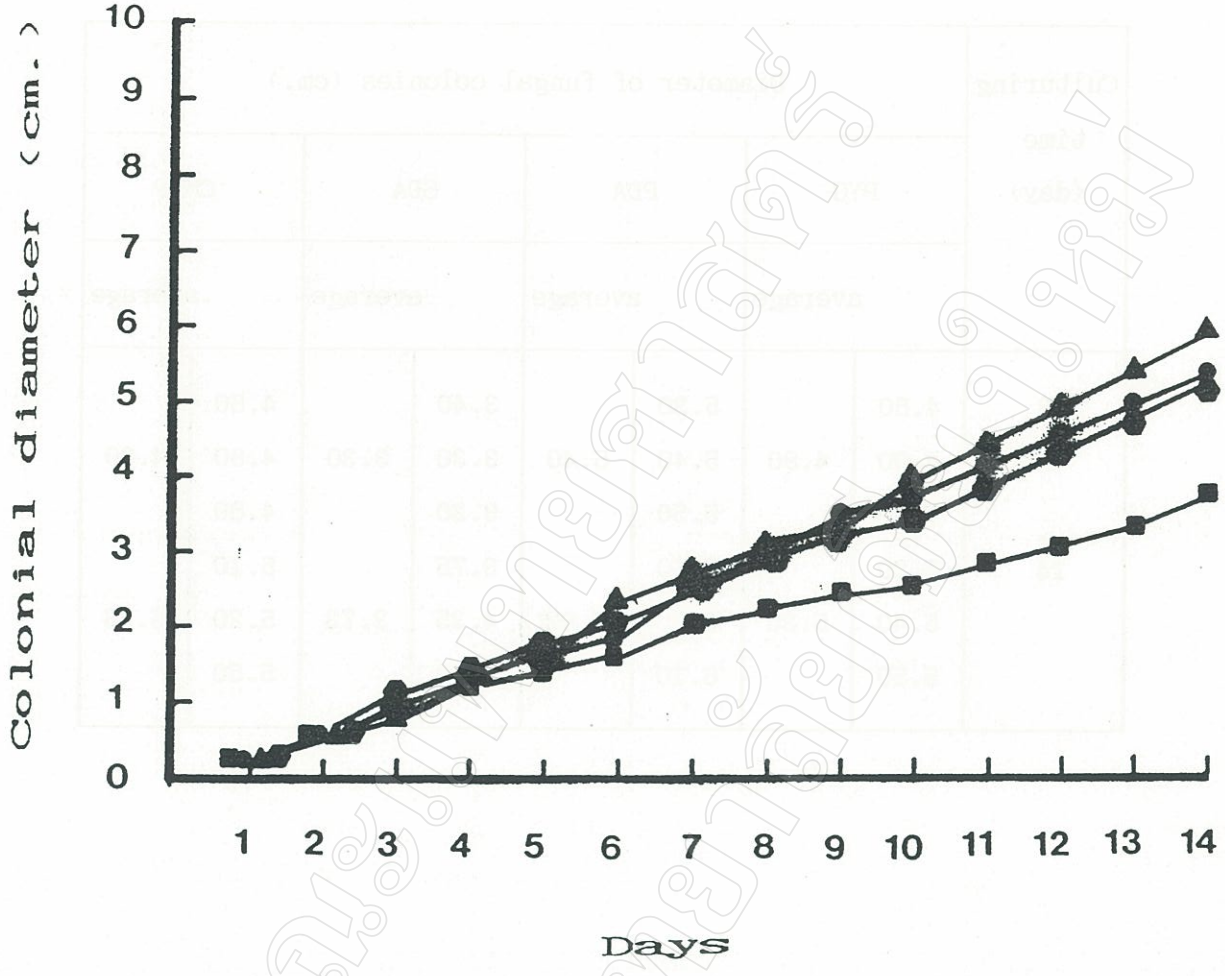

Figure 33. Growth of 10A-15W isolate of *Beauveria sp.* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬣) at room temperature determined by colonial diameters. Results represent the average of triplicate cultures.

Table 11 Growth of 10A-15W isolate of *Beauveria sp.* on different media at room temperature determined by dry weight of 11 days old cultures.

Fungus	Dry weight (milligrams)			
	PYG	PDA	SDA	CMDP
10A-15W isolate of *Beauveria sp.*	16.7 26.3	59.6 56.0	95.3 99.3	32.0 43.5
average	21.5	57.8	97.5	37.7

Table 12 Spore production of 10A-15W isolate of *Beauveria sp.* cultured on different media at room temperature.

Culturing time (day)	Spore enumeration (Spores/ml)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
5	1.95×10^5		2.97×10^5		4.25×10^4		9.50×10^4	
	1.30×10^5	1.62×10^5	1.92×10^5	2.45×10^5	2.25×10^4	3.25×10^4	1.20×10^5	1.07×10^5
7	1.15×10^5		3.22×10^5		1.12×10^5		7.75×10^4	
	1.20×10^5	1.17×10^5	2.52×10^5	2.87×10^5	1.17×10^5	1.15×10^5	1.00×10^5	8.87×10^4
14	5.99×10^6		2.95×10^5		1.57×10^5		1.55×10^6	
	4.55×10^6	5.27×10^6	1.47×10^5	2.21×10^5	2.12×10^5	1.85×10^5	3.05×10^6	2.30×10^6
24	4.63×10^7		3.67×10^7		1.09×10^7		2.00×10^7	
	3.79×10^7	4.21×10^7	4.45×10^7	4.06×10^7	8.45×10^6	9.67×10^6	2.86×10^7	2.43×10^7

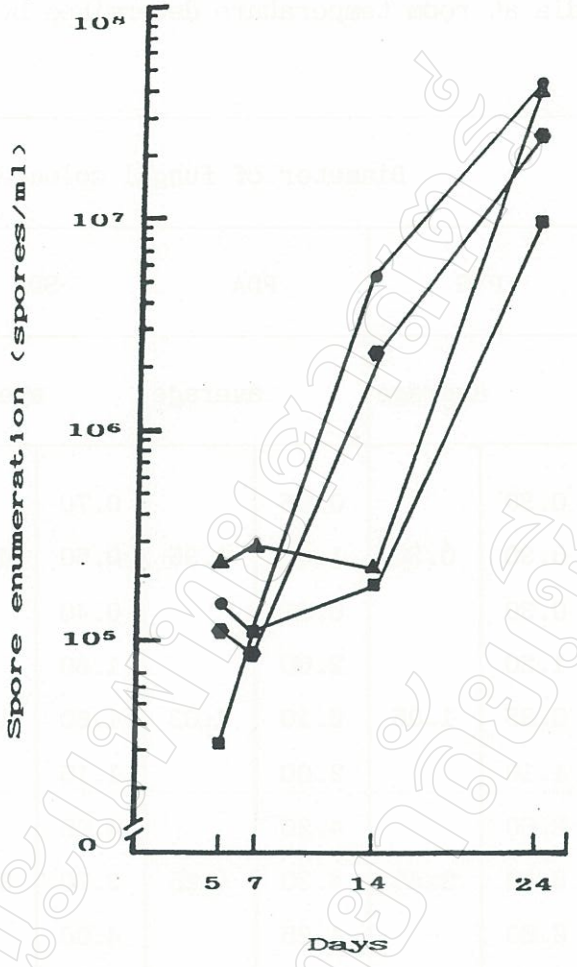

Figure 34. Spore production of 10A-15W isolate of *Beauveria sp.* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at different incubation time. Results represent the average of duplicate cultures.

Table 13 Growth of 29B-5W isolate of *Aspergillus niveus* on different media at room temperature determined by diameter of colonies.

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
1	0.30		0.95		0.70		0.65	
	0.30	0.30	1.00	0.95	0.50	0.53	0.70	0.66
	0.30		0.90.		0.40		0.65	
2	1.20		2.00		1.80		1.90	
	0.85	1.05	2.10	2.03	1.60	1.51	2.00	1.96
	1.10		2.00		1.15		2.00	
3	2.60		4.20		4.05		4.00	
	2.00	2.40	4.30	4.25	3.70	3.91	4.45	4.25
	2.60		4.25		4.00		4.30	
4	3.50		5.60		5.50		5.40	
	3.60	3.55	5.60	5.56	4.90	5.26	5.80	5.63
	3.55		5.50		5.40		5.70	
5	4.45		6.80		6.95		6.70	
	3.25	4.06	6.80	6.80	6.30	6.68	7.30	7.00
	4.50		6.80		6.80		7.00	
6	5.25		8.25		8.10		8.00	
	5.40	5.35	8.45	8.36	7.50	7.78	8.30	8.20
	5.40		8.40		7.75		8.30	

Table 13 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
7	6.40		8.70		9.00		9.00	
	6.40	6.40	9.00	8.70	9.00	8.90	9.00	9.00
	6.40		8.40		8.70		9.00	
8	7.25		9.00		9.00		9.00	
	7.25	7.30	9.00	9.00	9.00	9.00	9.00	9.00
	7.40		9.00		9.00		9.00	
9	7.80		9.00		9.00		9.00	
	7.80	7.80	9.00	9.00	9.00	9.00	9.00	9.00
	7.80		9.00		9.00		9.00	
10	8.30		9.00		9.00		9.00	
	8.30	8.30	9.00	9.00	9.00	9.00	9.00	9.00
	8.30		9.00		9.00		9.00	
11	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
12	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

Table 13 (Continued)

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
13	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
14	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

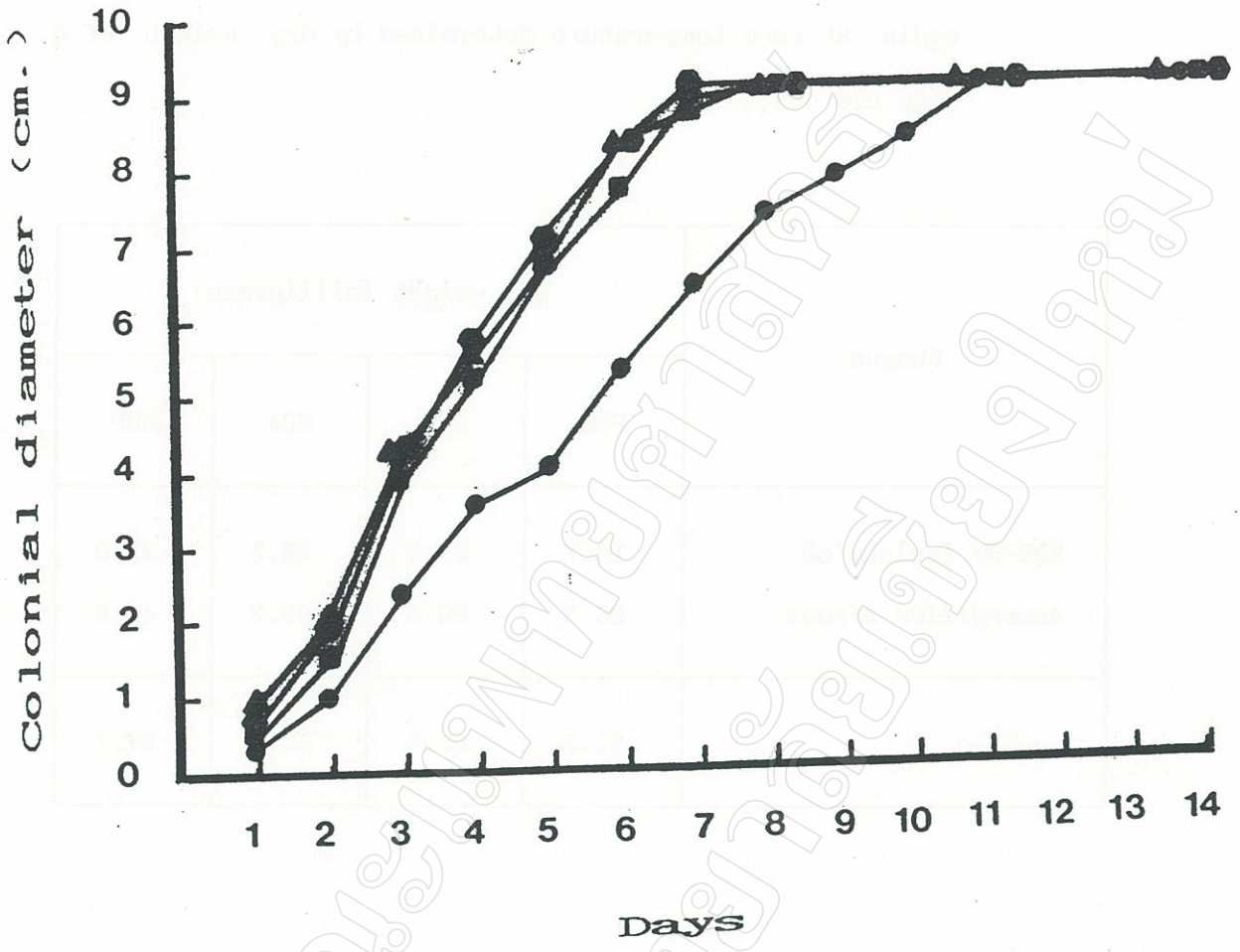

Figure 35. Growth of 29B-5W isolate of *Aspergillus niveus* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at room temperature determined by colonial diameters. Results represent the average of triplicate cultures.

Table 14 Growth of 29B-5W isolate of *Aspergillus niveus* on various media at room temperature determined by dry weight of 6 days old cultures.

Fungus	Dry weight (milligrams)			
	PYG	PDA	SDA	CMDP
29B-5W isolate of *Aspergillus niveus*	16.7 26.3	59.6 56.0	95.3 99.3	32.0 43.5
average	21.5	57.8	97.5	37.7

Table 15 Ascospore production of 29B-5W isolate of *Aspergillus niveus* cultured on different media at room temperature.

Culturing time (day)	Spore enumeration (Spores/ml)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
3	0		0		0		0	
	0	0	0	0	0	0	0	0
5	7.75×10^{4}		0		0		0	
	6.00×10^{4}	6.87×10^{4}	0	0	0	0	0	0
7	1.07×10^{5}		1.18×10^{6}		0		6.95×10^{5}	
	9.75×10^{4}	1.02×10^{5}	1.23×10^{6}	1.20×10^{6}	0	0	8.37×10^{5}	7.66×10^{5}
14	6.25×10^{4}		2.80×10^{7}		0		6.57×10^{6}	
	7.75×10^{4}	7.00×10^{4}	3.10×10^{7}	2.95×10^{7}	7.50×10^{3}	3.75×10^{3}	6.26×10^{6}	6.41×10^{6}
20	9.00×10^{4}		3.15×10^{7}		0		1.30×10^{7}	
	7.50×10^{4}	8.25×10^{4}	3.22×10^{7}	3.18×10^{7}	2.50×10^{3}	1.25×10^{3}	1.70×10^{7}	1.50×10^{7}
24	2.72×10^{5}		4.59×10^{7}		0		8.38×10^{7}	
	2.05×10^{5}	2.38×10^{5}	5.06×10^{7}	4.82×10^{7}	0	0	8.48×10^{7}	8.43×10^{7}

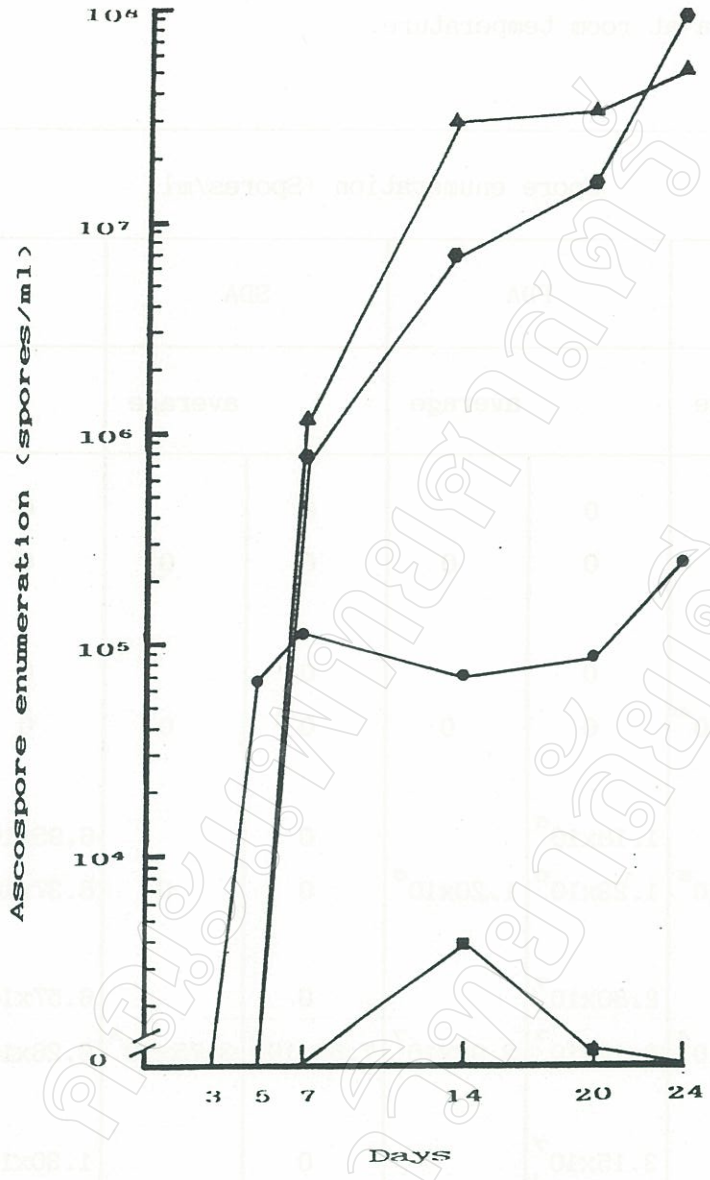

Figure 36. Ascospore production of 29B-5W isolate of *Aspergillus niveus* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at different incubation time. Results represent the average of duplicate cultures.

Table 16 Growth of 35C-1 isolate of *Aphanomyces sp.* on different media at room temperature determined by diameter of colonies.

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
1	2.80		3.00		3.00		2.95	
	2.80	2.76	3.10	3.06	3.10	3.03	3.20	3.05
	2.70		3.10		3.00		3.00	
2	5.20		6.00		5.65		5.80	
	5.20	5.18	6.00	5.98	5.70	5.66	5.90	5.85
	5.15		5.95		5.65		5.85	
3	7.60		9.00		8.35		8.50	
	7.60	7.56	9.00	9.00	8.50	8.40	9.00	8.83
	7.50		9.00		8.35		9.00	
4	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
5	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

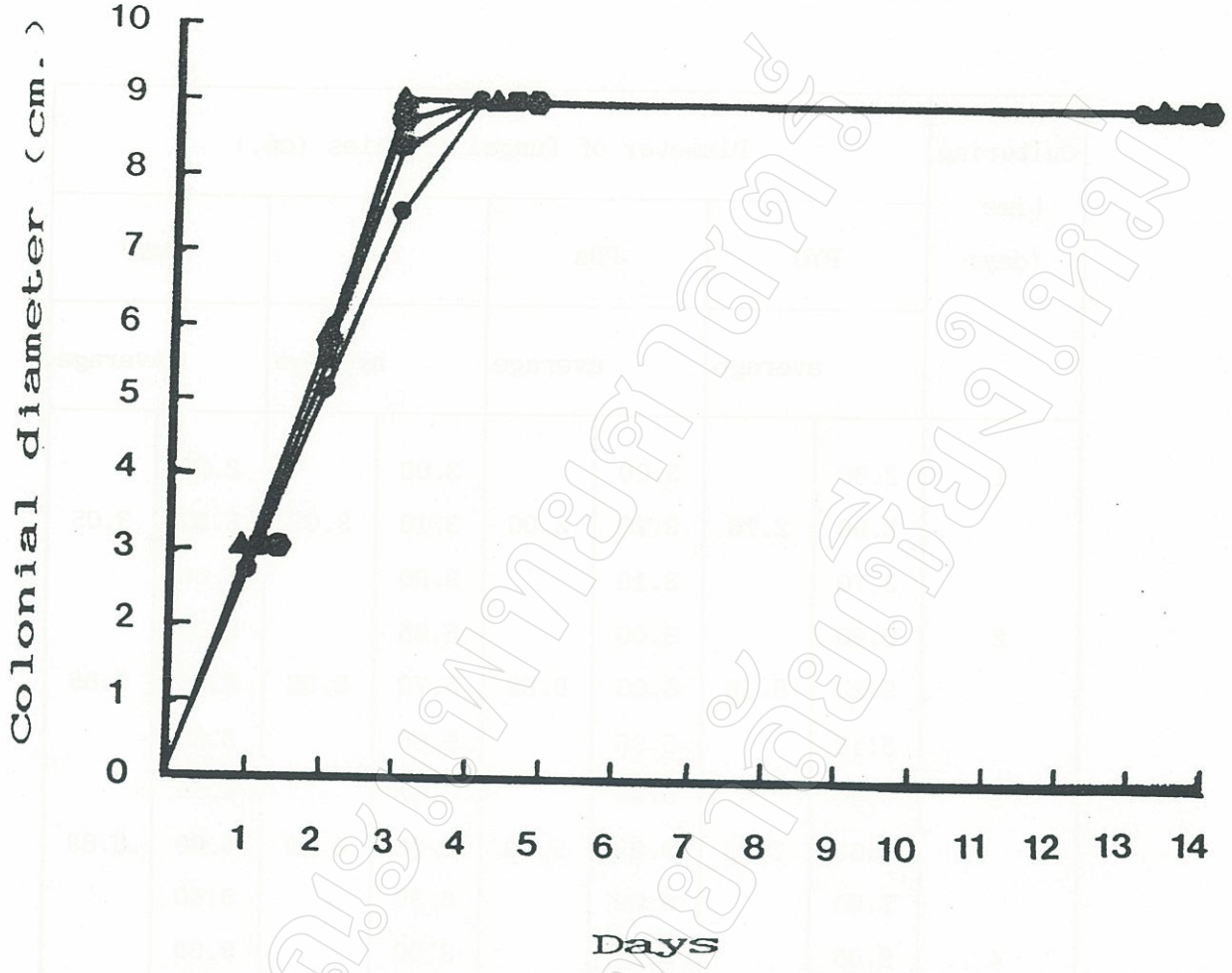

Figure 37. Growth of 35C-1 isolate of *Aphanomyces sp.* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬣) at room temperature determined by colonial diameters. Results represent the average of triplicate cultures.

Good growth was not obtained on PYG (19.1 mg dry weight) (Table 17).

For 36A-LK isolate of *Leptolegnia sp.*, the colony cultured on PDA, SDA and PYG reached 9 cm. in diameter within 3 day incubation at room temperature and 4 days on CMDP (Table 18 , Figure 38). But the growth on different media was not significantly different ($p>0.05$). This isolate cultured on SDA yielded highest dry weight (73.0mg) of 2 days old cultures ($p<0.05$) (Table 19). CMDP and PYG did not support the good growth of this isolate of fungus (24.0 mg and 17.6 mg dry weight, respectively).

Table 17 Growth of 35C-1 isolate of *Aphanomyces sp.* on various media at room temperature determined by dry weight of 2 days old cultures.

Fungus	Dry weight (milligrams)			
	PYG	PDA	SDA	CMDP
35C-1 isolate of *Aphanomyces sp.*	20.0 18.2	44.9 40.7	72.0 74.4	38.5 44.8
average	19.1	42.8	73.2	41.6

Table 18 Growth of 36A-LK isolate of *Leptolegnia sp.* on different media at room temperature determined by diameter of colonies.

Culturing time (day)	Diameter of fungal colonies (cm.)							
	PYG		PDA		SDA		CMDP	
		average		average		average		average
1	3.80		4.45		3.70		3.00	
	3.80	3.83	4.50	4.45	3.60	3.63	3.00	3.00
	3.90		4.40		3.60		3.00	
2	7.30		8.30		7.80		5.80	
	7.25	7.33	8.45	8.38	7.85	7.80	5.90	5.91
	7.45		8.40		7.75		6.05	
3	9.00		9.00		9.00		8.50	
	9.00	9.00	9.00	9.00	9.00	9.00	8.80	8.70
	9.00		9.00		9.00		8.80	
4	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
5	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

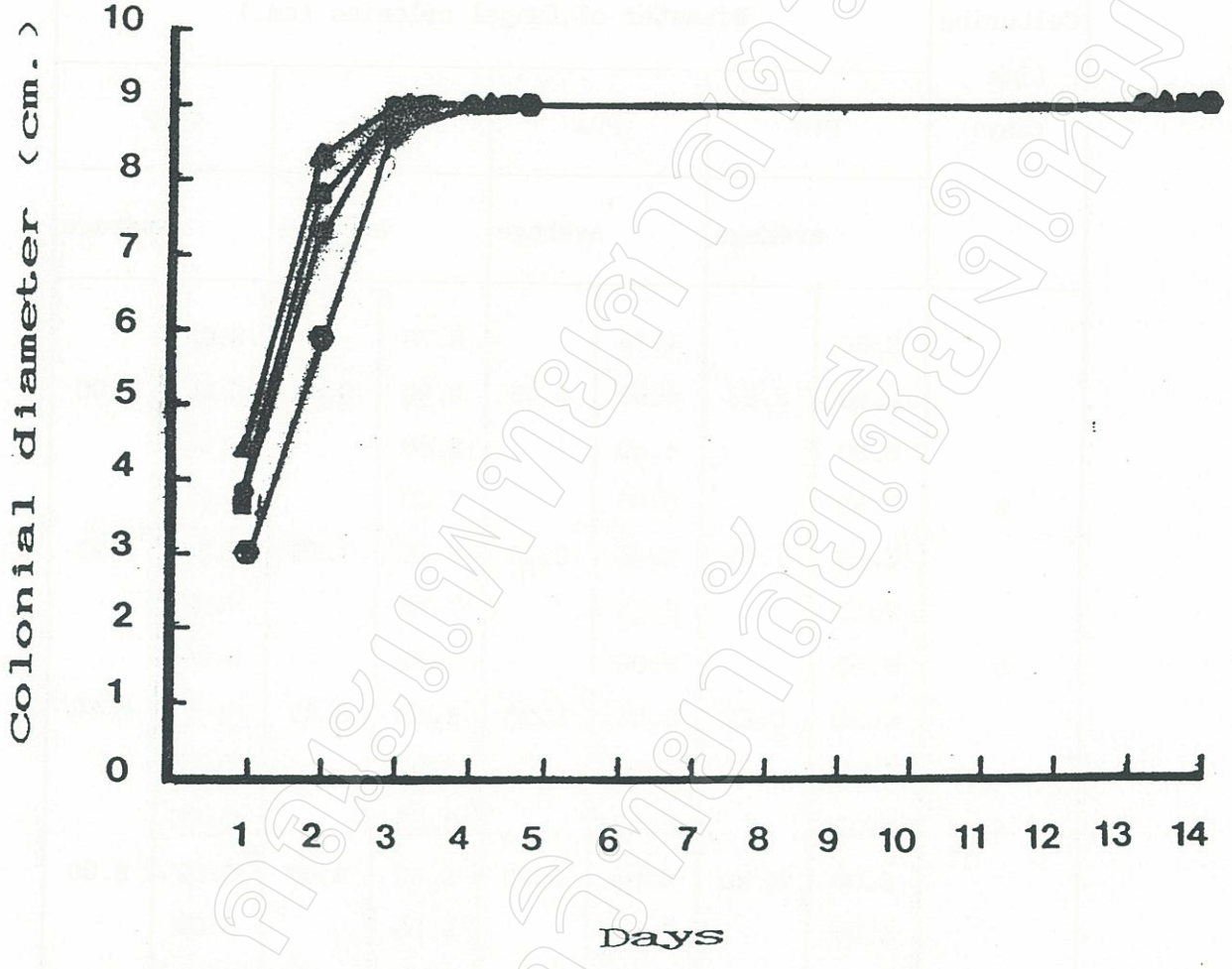

Figure 38. Growth of 36A-LK isolate of *Leptolegnia sp.* on different media; PYG (●), PDA (▲), SDA (■) and CMDP (⬢) at room temperature determined by colonial diameters. Results represent the average of triplicate cultures.

Table 19 Growth of 36A-LK isolate of *Leptolegnia sp.* on various media at room temperature determined by dry weight of 2 days old cultures.

Fungus	Dry weight (milligrams)			
	PYG	PDA	SDA	CMDP
36A-LK isolate of *Leptolegnia sp.*	15.8 19.5	47.2 51.7	67.1 78.9	21.9 26.2
average	17.6	49.4	73.0	24.0

2. Optimal conditions for growth and spore production of mosquito larvae killing fungi.

2.1. Temperature

Temperatures of water at the mosquito larvae collecting sites were recorded. They ranged from 18°C to 37°C. Hence, optimal temperatures for growths of mosquito larvae killing fungi were studied at 10, 20, 25 and 37 °C.

Growth of 12A-6 isolate of *Trichoderma viride* was determined by colonial diameters at different temperatures (Table 20, Figure 39-A). This fungus rapidly grew on CMDP agar at 25°C (Figure 39-B). At 37°C, this isolate did not grow after 2 weeks. It slowly grew when incubated at 10°C. The fungal growth at 20 °C and 25 °C was significantly higher than at 10 °C and 37°C ($p<0.01$). Its spore production correlated with fungal growth. At 25 °C, spore production was significantly higher than 20°C ($p<0.05$) until day 7 (Table 21, Figure 40-A). After that, there was no significant difference of the spore production at the two incubation temperatures (Figure 40-B). However, the spore production was still higher than those at 37 °C and 10 °C.

Growth of 10A-15W isolate of *Beauveria sp.* on CMDP was rapid at 25 °C (Table 22, Figure 41-A) and the colonial diameter was 4.53 cm. at day 14 and 7.21 cm. at day 24 of culturing

Table 20 Growth of 12A-6 isolate of *Trichoderma viride* on cornmeal dextrose peptone agar (CMDP) at various temperatures determined by colonial diameters.

Culturing time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
1	0		0.60		2.10		0.10	
	0	0	0.75	0.70	2.00	2.06	0.10	0.13
	0		0.75		2.10		0.20	
2	0		3.55		6.40		0.15	
	0	0	3.60	3.58	6.35	6.36	0.25	0.23
	0		3.60		6.35		0.30	
3	0.40		6.50		9.00		0.20	
	0.10	0.20	6.55	6.55	9.00	9.00	0.25	0.25
	0.10		6.60		9.00		0.30	
4	1.15		9.00		9.00		0.20	
	0.40	0.78	9.00	9.00	9.00	9.00	0.25	0.26
	0.80		9.00		9.00		0.35	
5	1.65		9.00		9.00		0.25	
	0.90	1.33	9.00	9.00	9.00	9.00	0.35	0.33
	1.45		9.00		9.00		0.40	
6	1.82		9.00		9.00		0.25	
	1.82	1.82	9.00	9.00	9.00	9.00	0.35	0.33
	1.82		9.00		9.00		0.40	

Table 20 (Continued)

Culturing time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
7	2.60		9.00		9.00		0.25	
	1.95	2.36	9.00	9.00	9.00	9.00	0.25	0.30
	2.55		9.00		9.00		0.40	
8	3.05		9.00		9.00		0.25	
	2.40	2.75	9.00	9.00	9.00	9.00	0.25	0.30
	2.80		9.00		9.00		0.40	
9	3.40		9.00		9.00		0.25	
	2.80	3.15	9.00	9.00	9.00	9.00	0.25	0.30
	3.25		9.00		9.00		0.40	
10	4.00		9.00		9.00		0.25	
	3.35	3.66	9.00	9.00	9.00	9.00	0.25	0.30
	3.65		9.00		9.00		0.40	
11	4.35		9.00		9.00		0.25	
	3.70	4.00	9.00	9.00	9.00	9.00	0.25	0.30
	3.95		9.00		9.00		0.40	
12	4.60		9.00		9.00		0.25	
	4.10	4.33	9.00	9.00	9.00	9.00	0.25	0.30
	4.30		9.00		9.00		0.40	

Table 20 (Continued)

Culturing time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		averagé		average
13	4.90		9.00		9.00		0.25	
	4.35	4.56	9.00	9.00	9.00	9.00	0.25	0.30
	4.45		9.00		9.00		0.40	
14	4.60		9.00		9.00		0.25	
	4.60	4.60	9.00	9.00	9.00	9.00	0.25	0.30
	4.60		9.00		9.00		0.40	
16	4.90		9.00		9.00		0.25	
	4.90	4.90	9.00	9.00	9.00	9.00	0.25	0.30
	4.90		9.00		9.00		0.40	
20	5.50		9.00		9.00		0.25	
	5.30	5.30	9.00	9.00	9.00	9.00	0.25	0.30
	5.10		9.00		9.00		0.40	
21	5.30		9.00		9.00		0.25	
	5.30	5.30	9.00	9.00	9.00	9.00	0.45	0.43
	5.30		9.00		9.00		0.60	
23	5.63		9.00		9.00		0.25	
	5.35	5.43	9.00	9.00	9.00	9.00	0.45	0.43
	5.30		9.00		9.00		0.60	
24	5.60		9.00		9.00		0.25	
	5.35	5.41	9.00	9.00	9.00	9.00	0.45	0.43
	5.30		9.00		9.00		0.60	

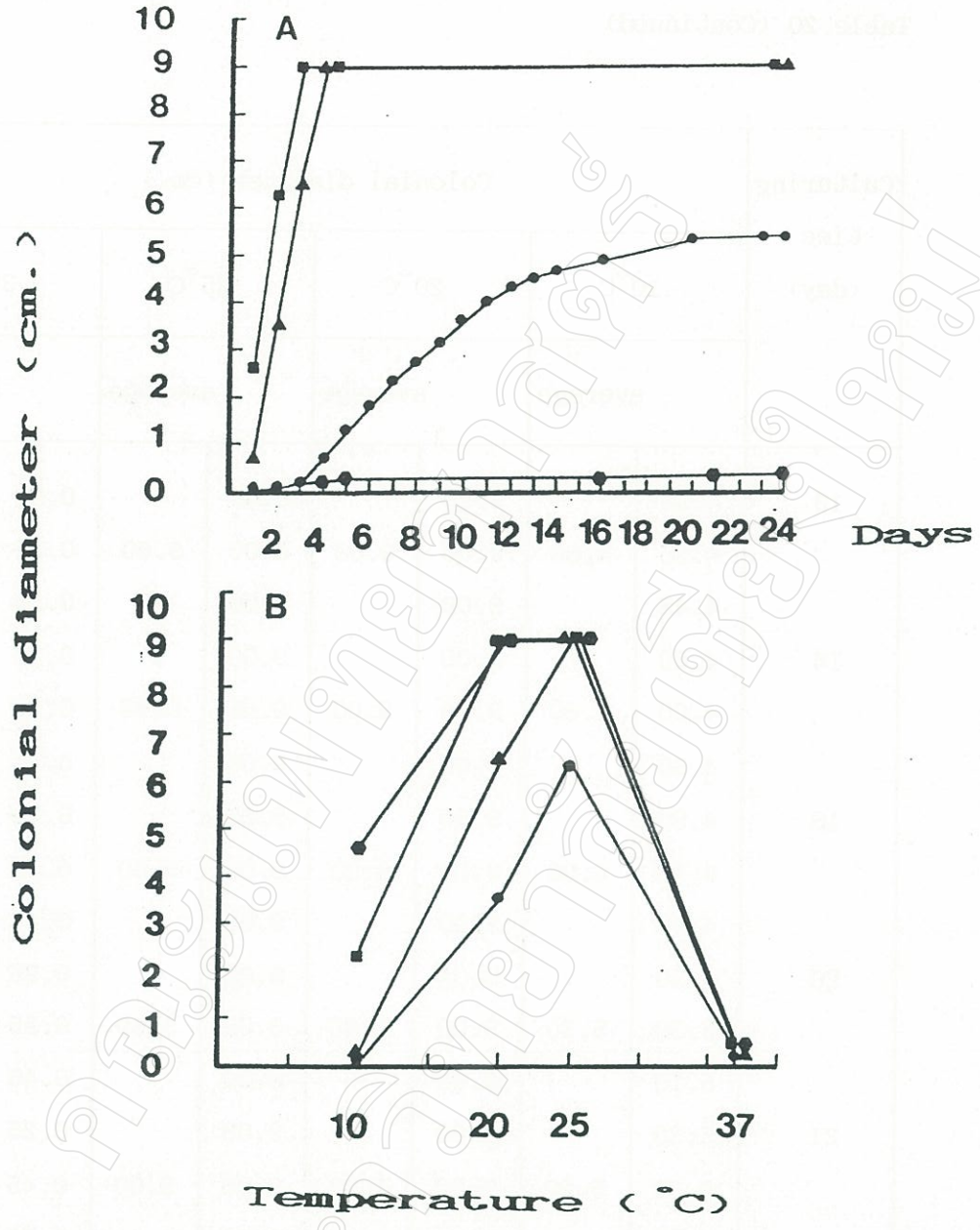

Figure 39. (A). Growth of 12A-6 isolate of *Trichoderma viride* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬢) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 12A-6 isolate in various temperatures determined by colonial diameters at day 2 (●), day 3 (▲), day 7 (■) and day 14 (⬢).

Table 21 Spore production of 12A-6 isolate of *Trichoderma viride* at various incubation temperatures.

Incubation time (day)	Spore enumeration (Spores/ml)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
3	0		4.44×10^{3}		2.48×10^{7}		2.00×10^{4}	
	0	0	3.33×10^{3}	3.88×10^{3}	2.93×10^{7}	2.71×10^{7}	2.00×10^{4}	2.00×10^{4}
5	0		1.36×10^{7}		9.05×10^{7}		5.00×10^{4}	
	0	0	1.43×10^{7}	1.39×10^{7}	1.15×10^{8}	1.02×10^{8}	3.00×10^{4}	4.00×10^{4}
7	0		1.69×10^{8}		2.53×10^{8}		5.00×10^{4}	
	0	0	1.84×10^{8}	1.76×10^{8}	2.40×10^{8}	2.46×10^{8}	3.00×10^{4}	4.00×10^{4}
14	0		4.13×10^{8}		3.67×10^{8}		1.25×10^{4}	
	0	0	4.51×10^{8}	4.32×10^{8}	3.87×10^{8}	3.77×10^{8}	1.00×10^{4}	1.12×10^{4}
20	0		3.78×10^{8}		3.57×10^{8}		0	
	0	0	2.96×10^{8}	3.37×10^{8}	3.42×10^{8}	3.49×10^{8}	1.00×10^{4}	5.00×10^{3}
24	3.33×10^{3}		3.57×10^{8}		4.06×10^{8}		0	
	0	1.66×10^{3}	3.16×10^{8}	3.37×10^{8}	3.65×10^{8}	3.85×10^{8}	0	0

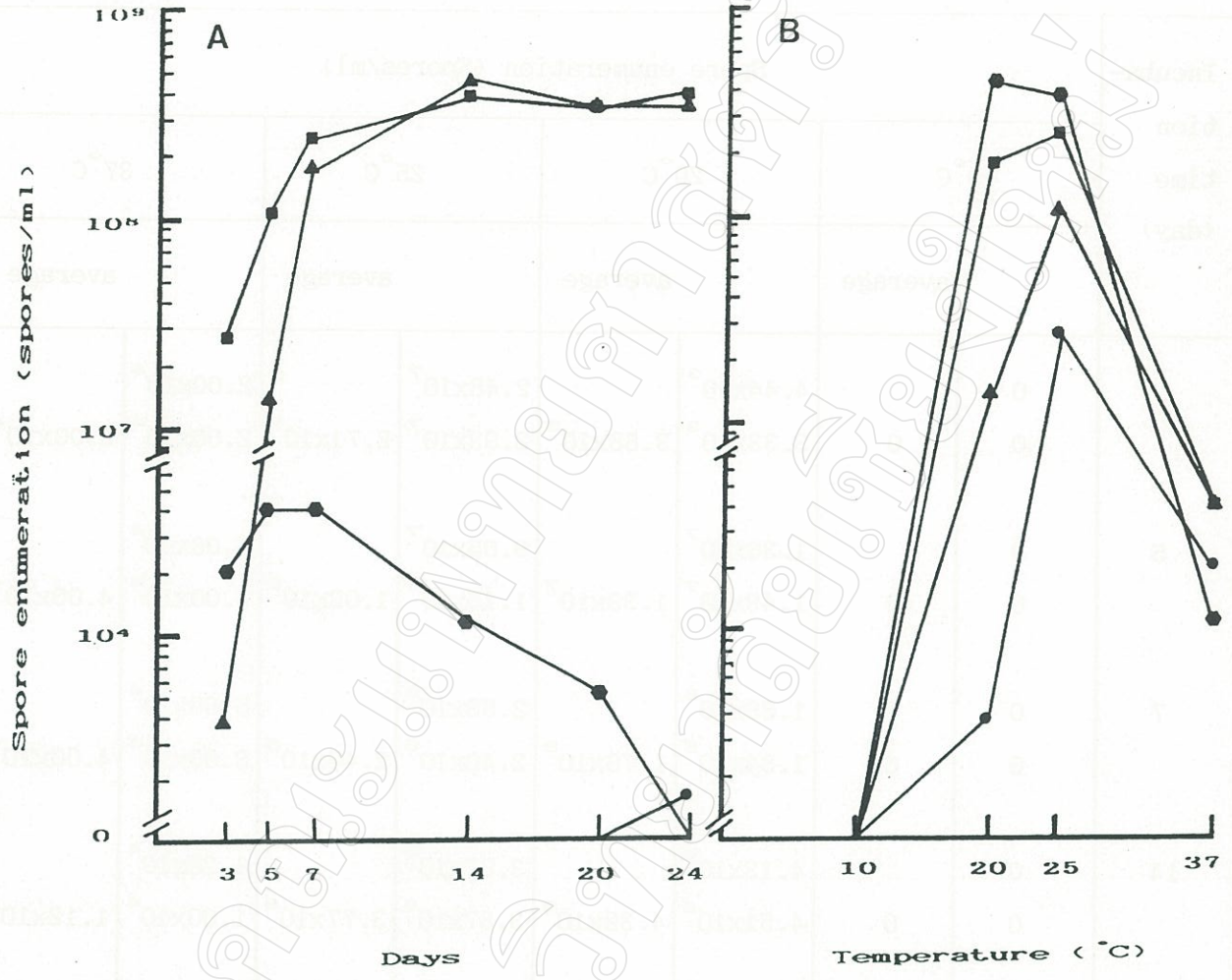

Figure 40. (A). Spore production of 12A-6 isolate of *Trichoderma viride* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬣). Results represent the average of triplicate cultures.

(B). Spore production of 12A-6 isolate at day 3 (●), day 5 (▲), day 7 (■) and day 14 (⬣) in various temperatures.

Table 22 Growth of 10A-15W isolate of *Beauveria sp.* on cornmeal dextrose peptone agar (CMDP) at various temperatures determined by colonial diameters.

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
1	0		0.05		0.10		0	
	0	0	0.05	0.05	0.10	0.10	0	0
	0		0.05		0.10		0	
2	0		0.35		0.50		0	
	0	0	0.35	0.36	0.50	0.50	0	0
	0		0.40		0.50		0	
3	0.10		0.60		0.85		0	
	0.10	0.10	0.60	0.63	0.90	0.88	0	0
	0.10		0.70		0.90		0	
4	0.15		0.85		1.10		0	
	0.15	0.15	0.80	0.91	1.10	1.11	0	0
	0.15		1.10		1.10		0	
5	0.20		1.10		1.40		0	
	0.20	0.20	1.05	1.15	1.50	1.46	0	0
	0.20		1.30		1.50		0	
6	0.25		1.30		1.80		0	
	0.25	0.25	1.25	1.35	1.80	1.80	0	0
	0.25		1.50		1.80		0	

Table 22 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
7	0.35		1.50		2.00		0	
	0.40	0.35	1.45	1.55	2.10	2.10	0	0
	0.30		1.70		2.20		0	
8	0.45		1.72		2.20		0	
	0.50	0.45	1.72	1.72	2.40	2.36	0	0
	0.40		1.72		2.50		0	
9	0.50		2.00		2.75		0	
	0.60	0.53	2.00	2.00	2.70	2.76	0	0
	0.50		2.00		2.85		0	
10	0.55		2.20		3.05		0	
	0.55	0.53	2.20	2.30	3.10	3.11	0	0
	0.50		2.50		3.20		0	
11	0.65		2.50		3.40		0	
	0.60	0.61	2.50	2.50	3.40	3.40	0	0
	0.60		2.50		3.40		0	
12	0.65		2.90		3.80		0	
	0.70	0.68	2.70	2.93	3.80	3.80	0	0
	0.70		3.20		3.80		0	

Table 22 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
13	0.65		3.15		4.10		0	
	0.75	0.70	3.15	3.15	4.30	4.20	0	0
	0.70		3.15		4.20		0	
14	0.70		3.30		4.20		0	
	0.70	0.70	3.45	3.51	4.70	4.53	0	0
	0.70		3.80		4.70		0	
15	0.80		3.80		4.90		0	
	0.80	0.80	3.80	3.80	5.15	5.05	0	0
	0.80		3.80		5.10		0	
16	0.80		4.20		5.20		0	
	0.80	0.80	4.20	4.20	5.40	5.31	0	0
	0.80		4.20		5.35		0	
17	0.89		4.50		5.50		0	
	0.89	0.89	4.60	4.65	5.80	5.68	0	0
	0.89		4.85		5.75		0	
18	0.90		4.90		5.90		0	
	0.90	0.90	4.90	4.90	6.30	6.18	0	0
	0.90		4.90		6.35		0	

Table 22 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
19	0.92		5.30		6.25		0	
	0.92	0.92	5.30	5.30	6.65	6.46	0	0
	0.92		5.30		6.50		0	
20	1.05		5.60		6.55		0	
	1.10	1.05	5.60	5.60	6.80	6.78	0	0
	1.00		5.60		7.00		0	
21	1.10		5.60		6.85		0	
	1.10	1.10	6.10	6.00	7.10	7.08	0	0
	1.10		6.30		7.30		0	
22	1.15		6.20		6.90		0	
	1.15	1.15	6.20	6.20	6.45	6.83	0	0
	1.15		6.20		7.15		0	
23	1.25		6.50		7.05		0	
	1.25	1.23	6.50	6.50	7.25	7.23	0	0
	1.20		6.50		7.40		0	
24	1.30		6.40		7.10		0	
	1.35	1.31	7.00	6.86	7.15	7.21	0	0
	1.30		7.20		7.40		0	

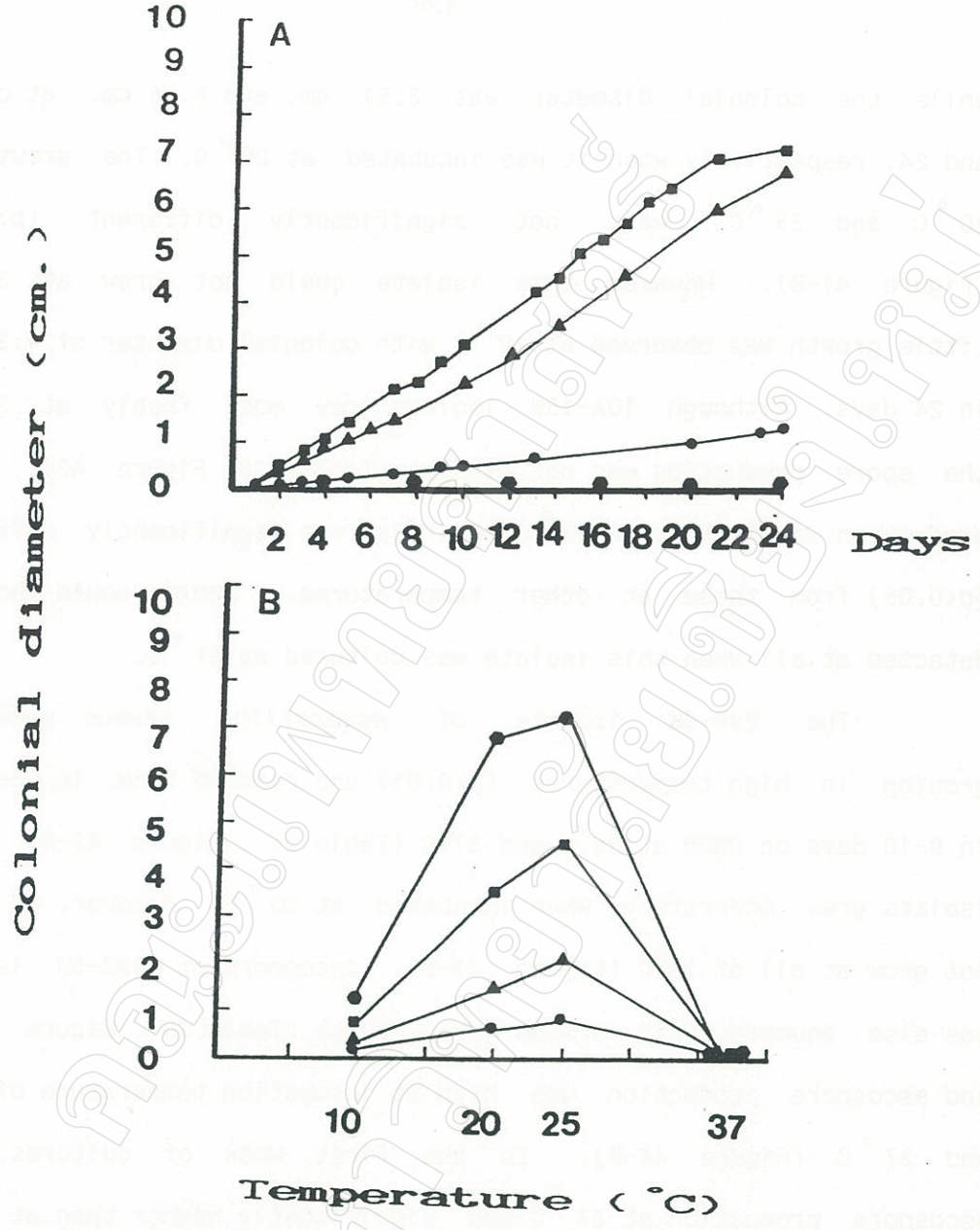

Figure 41. (A). Growth of 10A-15W isolate of *Beauveria sp.* in various temperatures; 10 °C (●), 20 °C (▲), 25 °C (■) and 37 °C (⬢) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 10A-15W isolate in various temperatures determined by colonial diameters at day 3 (●), day 7 (▲), day 14 (■) and day 24 (⬢).

while the colonial diameter was 3.51 cm. and 6.86 cm. at day 14 and 24, respectively when it was incubated at 20 °C. The growth at 20 °C and 25 °C were not significantly different ($p>0.05$) (Figure 41-B). However, this isolate could not grow at 37 °C. Little growth was observed at 10 °C with colonial diameter of 1.31 cm. in 24 days. Although 10A-15W isolate grew most fastly at 25 °C, the spore production was not highest (Table 23, Figure 42). Spore production was highest at 20 °C and this was significantly different ($p<0.05$) from those at other temperatures. Spore could not be detected at all when this isolate was cultured at 37 °C.

The 29B-5W isolate of *Aspergillus niveus* preferred growing in high temperatures ($p<0.01$) and reached 9 cm. in diameter in 9-10 days on CMDP at 25°C and 37°C (Table 24 , Figure 43-A). This isolate grew moderately when incubated at 20 °C. However, it could not grow at all at 10°C (Figure 43-B). Ascospore of 29B-5W isolate was also enumerated at various temperatures (Table 25 , Figure 44-A) and ascospore production was high at incubation temperature of 25°C and 37 °C (Figure 44-B). In the first week of cultures, the ascospore production at 37 °C was significantly higher than at 25 °C but after a week later, ascospore production at 25 °C was significant the highest ($p<0.05$). When this isolate was cultured at 20 °C, the ascospore production was lower than at incubation temperatures above. However, the ascospore production could not be detected when incubated at 10°C.

The 35C-1 isolate of *Aphanomyces sp.* cultured on

Table 23 Spore production of 10A-15W isolate of *Beauveria sp.* at various incubation temperatures.

Incubation time (day)	Spore enumeration (Spores/ml)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
3	3.50×10^{5}		4.50×10^{5}		9.75×10^{4}		0	
	3.60×10^{5}	3.55×10^{5}	1.70×10^{5}	3.10×10^{5}	7.00×10^{4}	8.37×10^{4}	0	0
5	1.40×10^{5}		1.32×10^{6}		2.23×10^{6}		0	
	9.00×10^{4}	1.15×10^{5}	1.62×10^{6}	1.47×10^{6}	1.81×10^{6}	2.02×10^{6}	0	0
7	0		7.40×10^{6}		2.20×10^{6}		0	
	2.00×10^{4}	1.00×10^{4}	6.30×10^{6}	6.85×10^{6}	3.13×10^{6}	2.66×10^{6}	0	0
14	1.40×10^{4}		1.31×10^{7}		6.90×10^{6}		0	
	1.60×10^{4}	1.50×10^{4}	1.58×10^{7}	1.44×10^{7}	4.40×10^{6}	5.65×10^{6}	0	0
20	2.50×10^{4}		1.50×10^{7}		6.50×10^{6}		0	
	2.75×10^{4}	2.62×10^{4}	1.50×10^{7}	1.50×10^{7}	7.75×10^{6}	7.12×10^{6}	0	0
24	7.00×10^{4}		2.01×10^{7}		5.65×10^{6}		0	
	3.00×10^{4}	5.00×10^{4}	2.20×10^{7}	2.10×10^{7}	7.20×10^{6}	6.42×10^{6}	0	0

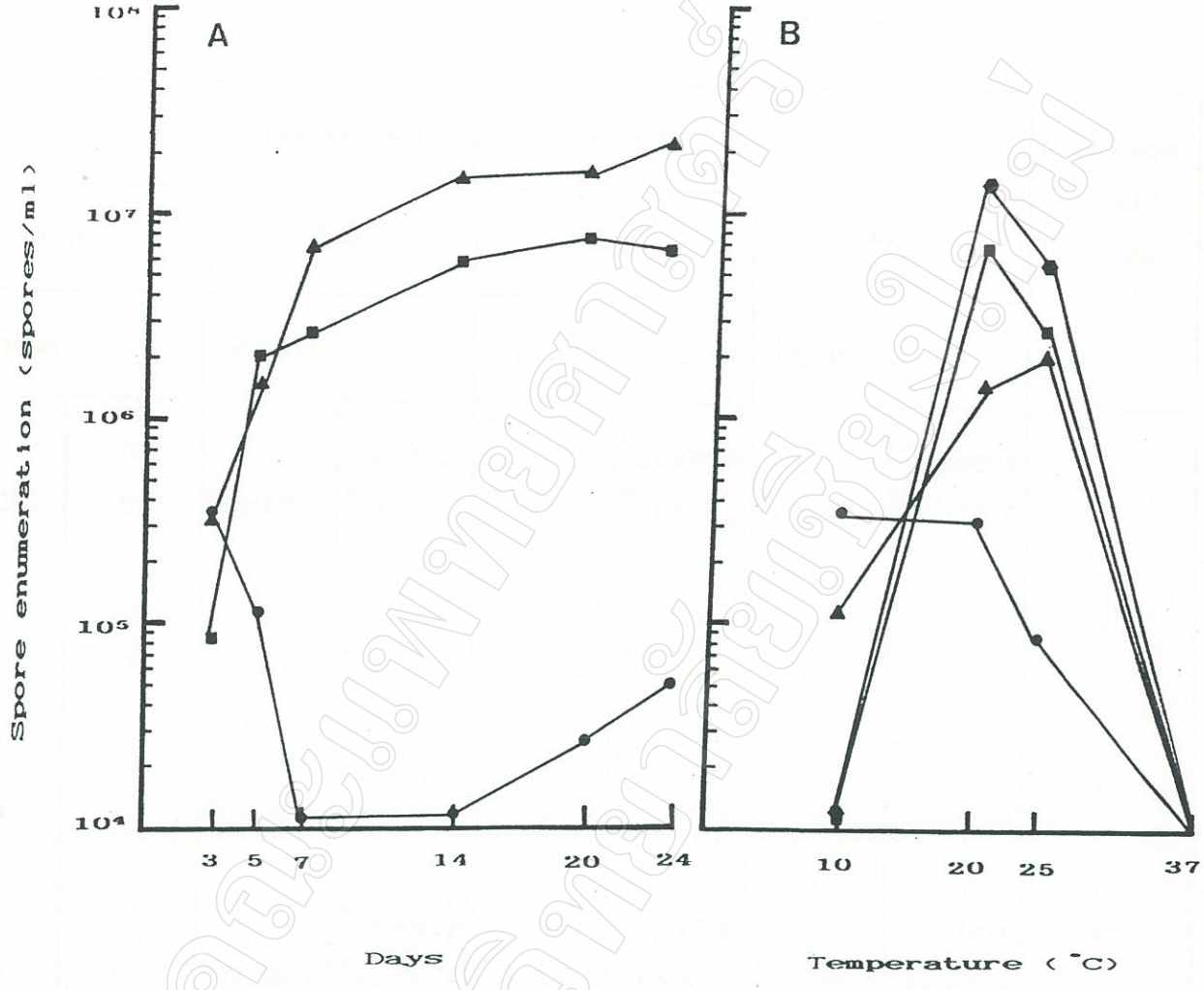

Figure 42. (A). Spore production of 10A-15W isolate of *Beauveria sp.* in various temperatures; 10 °C (●), 20 °C (▲), 25 °C (■) and 37 °C (⬢). Results represent the average of triplicate cultures.

(B). Spore production of 10A-15W isolate at day 3 (●), day 5 (▲), day 7 (■) and day 14 (⬢) in various temperatures.

Table 24 Growth of 29B-5W isolate of *Aspergillus niveus* on cornmeal dextrose peptone agar (CMDP) at various temperatures determined by colonial diameters.

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
1	0		0		0		0	
	0	0	0	0	0	0	0	0
	0		0		0		0	
2	0		0.30		1.00		1.80	
	0	0	0.30	0.30	1.00	1.00	1.80	1.76
	0		0.30		1.00		1.70	
3	0		0.90		2.00		3.20	
	0	0	0.85	0.90	2.10	2.10	3.20	3.30
	0		0.95		2.20		3.50	
4	0		1.20		3.10		4.30	
	0	0	1.30	1.33	3.10	3.20	4.40	4.43
	0		1.50		3.40		4.60	
5	0		2.10		4.70		6.10	
	0	0	2.30	2.23	4.90	4.73	6.10	6.00
	0		2.30		4.60		5.80	
6	0		3.10		6.00		7.30	
	0	0	3.00	3.10	6.00	6.00	7.30	7.40
	0		3.20		6.00		7.60	

Table 24 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
7	0		4.00		7.40		8.00	
	0	0	4.10	4.10	7.50	7.50	8.20	8.16
	0		4.20		7.60		8.30	
8	0		4.70		8.40		8.20	
	0	0	4.60	4.56	8.40	8.40	8.30	8.40
	0		4.40		8.40		8.70	
9	0		5.00		9.00		8.90	
	0	0	4.90	5.03	9.00	9.00	8.80	8.80
	0		5.20		9.00		8.70	
10	0		5.30		9.00		9.00	
	0	0	5.70	5.46	9.00	9.00	9.00	9.00
	0		5.40		9.00		9.00	
11	0		5.95		9.00		9.00	
	0	0	5.90	5.90	9.00	9.00	9.00	9.00
	0		5.85		9.00		9.00	
12	0		6.20		9.00		9.00	
	0	0	6.30	6.30	9.00	9.00	9.00	9.00
	0		6.40		9.00		9.00	

Table 24 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
13	0		6.70		9.00		9.00	
	0	0	6.90	6.73	9.00	9.00	9.00	9.00
	0		6.60		9.00		9.00	
14	0		7.20		9.00		9.00	
	0	0	7.10	7.13	9.00	9.00	9.00	9.00
	0		7.10		9.00		9.00	
15	0		7.00		9.00		9.00	
	0	0	7.40	7.10	9.00	9.00	9.00	9.00
	0		6.90		9.00		9.00	
16	0		7.50		9.00		9.00	
	0	0	7.50	7.50	9.00	9.00	9.00	9.00
	0		7.50		9.00		9.00	
17	0		7.50		9.00		9.00	
	0	0	7.40	7.56	9.00	9.00	9.00	9.00
	0		7.80		9.00		9.00	
18	0		7.60		9.00		9.00	
	0	0	7.60	7.70	9.00	9.00	9.00	9.00
	0		7.90		9.00		9.00	

Table 24 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
19	0		7.70		9.00		9.00	
	0	0	7.70	7.70	9.00	9.00	9.00	9.00
	0		7.70		9.00		9.00	
20	0		7.90		9.00		9.00	
	0	0	7.80	7.90	9.00	9.00	9.00	9.00
	0		8.00		9.00		9.00	
21	0		8.00		9.00		9.00	
	0	0	8.00	8.00	9.00	9.00	9.00	9.00
	0		8.00		9.00		9.00	
22	0		8.00		9.00		9.00	
	0	0	8.00	8.00	9.00	9.00	9.00	9.00
	0		8.00		9.00		9.00	
23	0		8.00		9.00		9.00	
	0	0	8.00	8.00	9.00	9.00	9.00	9.00
	0		8.00		9.00		9.00	
24	0		8.00		9.00		9.00	
	0	0	8.00	8.10	9.00	9.00	9.00	9.00
	0		8.30		9.00		9.00	

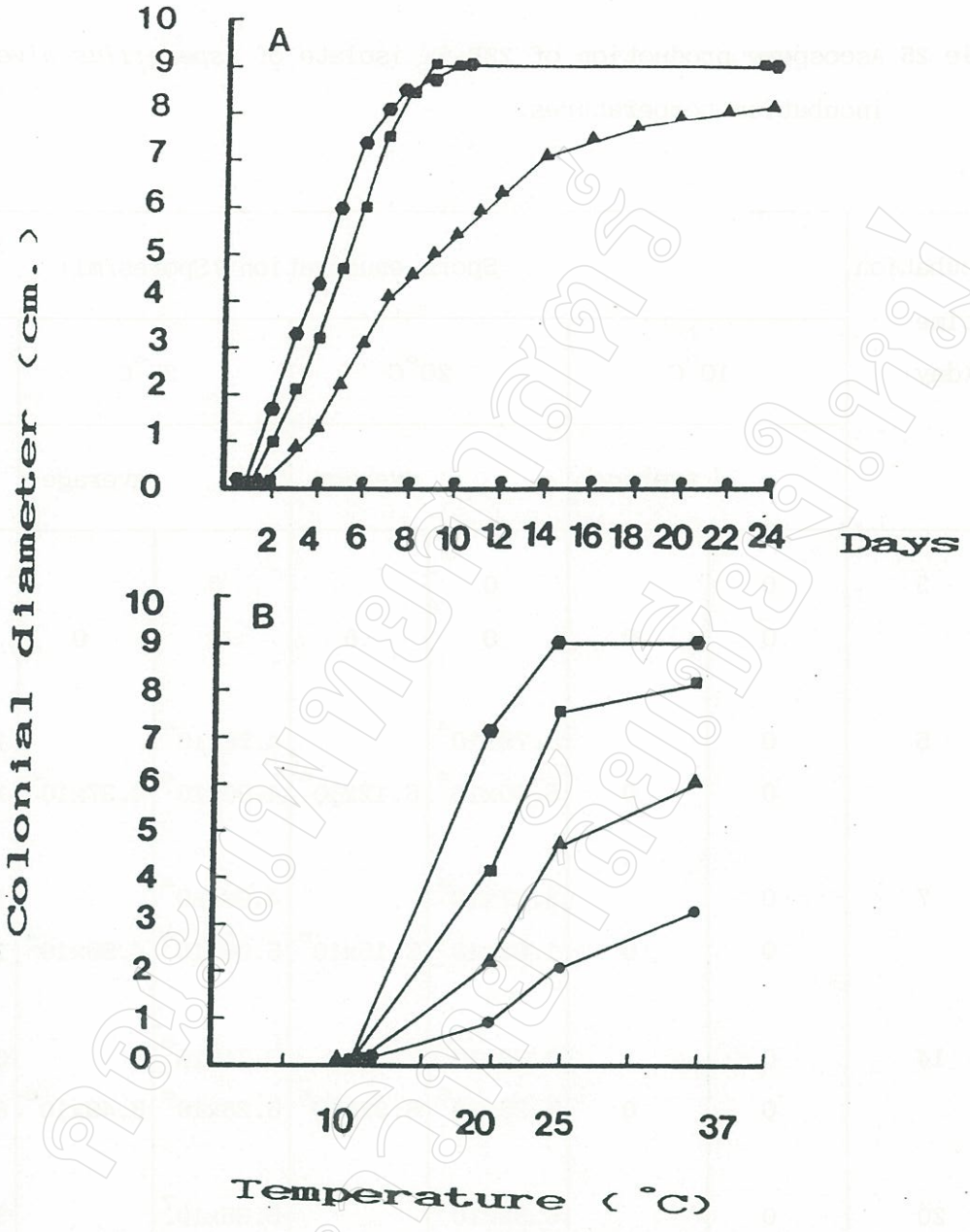

Figure 43. (A). Growth of 29B-5W isolate of *Aspergillus niveus* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬢) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 29B-5W isolate in various temperatures determined by colonial diameters at day 3 (●), day 5 (▲), day 7 (■), day 14 (⬢)

Table 25 Ascospore production of 29B-5W isolate of *Aspergillus niveus* at various incubation temperatures.

Incubation time (day)	Spore enumeration (Spores/ml)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
3	0		0		0		0	
	0	0	0	0	0	0	0	0
5	0		6.75×10^4		4.75×10^4		1.55×10^5	
	0	0	5.50×10^4	6.12×10^4	4.00×10^4	4.37×10^4	1.72×10^5	1.63×10^5
7	0		3.27×10^5		4.00×10^4		1.60×10^6	
	0	0	1.02×10^5	2.15×10^5	5.00×10^4	4.50×10^4	1.16×10^6	1.38×10^6
14	0		6.60×10^5		6.71×10^6		6.88×10^6	
	0	0	7.25×10^5	6.92×10^5	6.28×10^6	6.49×10^6	6.91×10^6	6.89×10^6
20	0		6.52×10^5		5.35×10^7		3.77×10^7	
	0	0	7.82×10^5	7.17×10^5	5.99×10^7	5.67×10^7	3.97×10^7	3.87×10^7
24	0		2.12×10^6		1.31×10^8		7.25×10^7	
	0	0	1.80×10^6	1.96×10^6	1.23×10^8	1.27×10^8	6.50×10^7	6.87×10^7

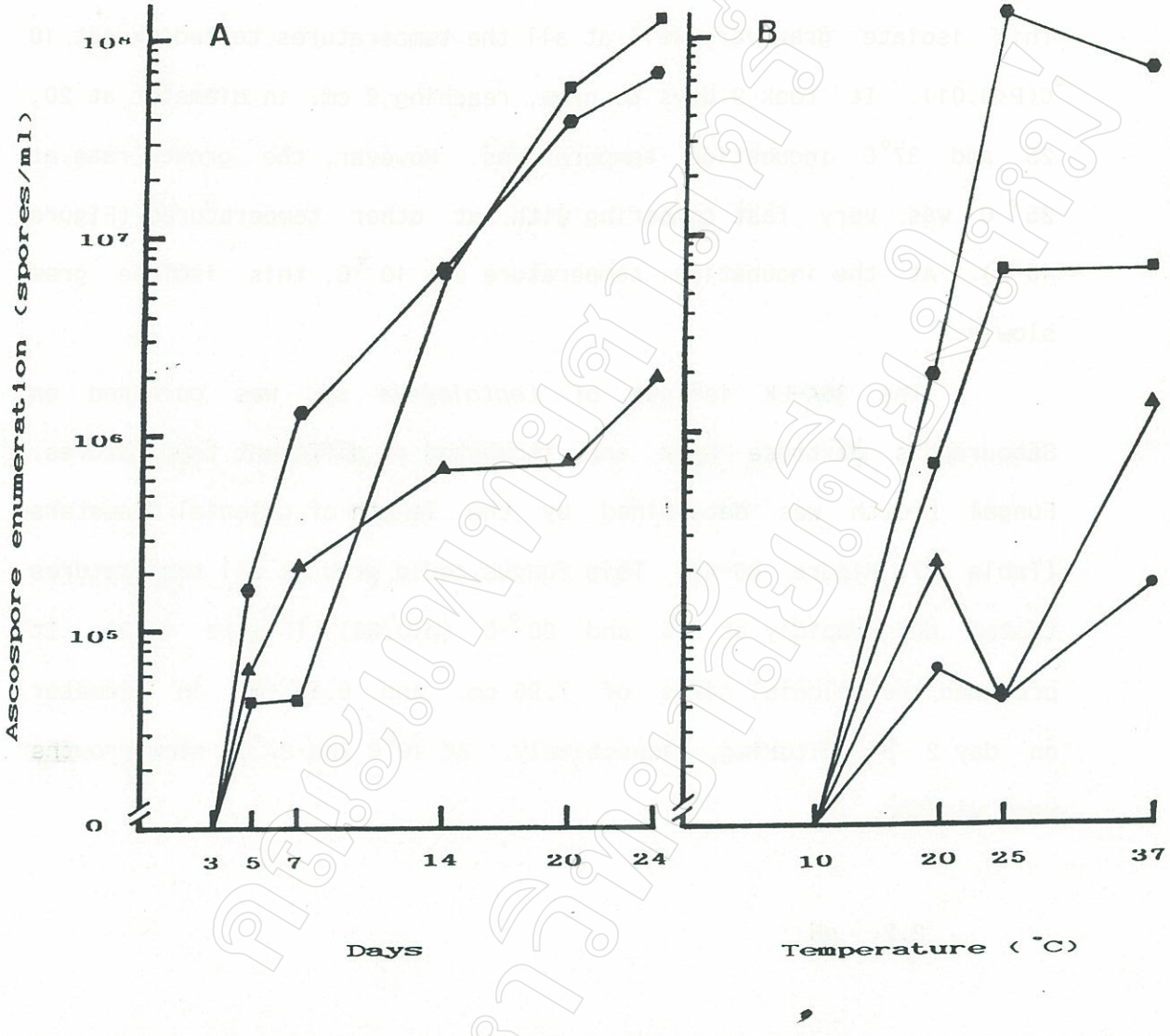

Figure 44. (A). Ascospore production of 29B-5W isolate of *Aspergillus niveus* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬢). Results represent the average of triplicate cultures.

(B). Ascospore production of 29B-5W isolate at day 5 (●), day 7 (▲), day 14 (■) and day 24 (⬢) in various temperatures.

Sabouraud's dextrose agar was incubated at various incubation temperatures and its growth was determined (Table 26 , Figure 45-A). This isolate grew very well at all the temperatures tested except 10 °C($P<0.01$). It took 9 days to grow, reaching 9 cm. in diameter at 20, 25 and 37°C incubation temperatures. However, the growth rate at 25 ° C was very fast comparing with at other temperatures (Figure 45-B). At the incubation temperature of 10 °C, this isolate grew slowly.

The 36A-LK isolate of *Leptolegnia sp.* was cultured on Sabouraud's dextrose agar and incubated at different temperatures. Fungal growth was determined by the length of colonial diameters (Table 27, Figure 46-A). This fungus could grow at all temperatures tested and rapidly at 25 and 20 ° C ($p<0.05$) (Figure 46-B). It produced the colonial sizes of 7.96 cm. and 6.16 cm. in diameter on day 2 of culturing, respectively. At 10°C and 37°C, slow growths were yielded.

2.2. pH

The pH of water at the mosquito larvae collecting sites was recorded. It ranged from pH 5.5 to pH 7.7 Hence, the growth of mosquito larvae killing fungi was investigated at pH 4, 5, 6, 7, and 8.

Growth of 12A-6 isolate of *Trichoderma viride*

Table 26 Growth of 35C-1 isolate of *Aphanomyces sp.* on Sabouraud's dextrose agar (SDA) at various temperature determined by colonial diameters.

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
1	0.90		2.05		2.90		2.40	
	0.90	0.93	2.15	2.16	2.85	2.90	2.40	2.38
	1.00		2.30		2.95		2.35	
2	1.70		4.10		6.25		5.15	
	1.60	1.66	4.20	4.21	6.30	6.31	5.25	5.11
	1.70		4.35		6.40		4.95	
3	2.25		5.75		8.80		6.70	
	2.25	2.26	6.00	5.91	8.80	8.76	6.75	6.56
	2.30		6.00		8.70		6.25	
4	2.85		8.00		9.00		8.25	
	2.80	2.83	7.90	7.90	9.00	9.00	8.40	8.33
	2.85		7.80		9.00		8.35	
5	3.60		8.60		9.00		8.90	
	3.50	3.53	8.80	8.63	9.00	9.00	8.80	8.90
	3.50		8.50		9.00		9.00	
6	4.00		9.00		9.00		8.80	
	4.00	4.00	9.00	8.93	9.00	9.00	9.00	8.93
	4.00		8.80		9.00		9.00	

Table 26 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
7	4.60		9.00		9.00		9.00	
	4.55	4.55	9.00	8.93	9.00	9.00	9.00	9.00
	4.50		8.80		9.00		9.00	
8	5.20		9.00		9.00		9.00	
	5.20	5.13	9.00	8.93	9.00	9.00	9.00	9.00
	5.00		8.80		9.00		9.00	
9	5.75		9.00		9.00		9.00	
	5.80	5.71	9.00	9.00	9.00	9.00	9.00	9.00
	5.60		9.00		9.00		9.00	
10	6.40		9.00		9.00		9.00	
	6.40	6.33	9.00	9.00	9.00	9.00	9.00	9.00
	6.20		9.00		9.00		9.00	
11	7.10		9.00		9.00		9.00	
	7.00	6.96	9.00	9.00	9.00	9.00	9.00	9.00
	6.80		9.00		9.00		9.00	
12	7.30		9.00		9.00		9.00	
	7.40	7.26	9.00	9.00	9.00	9.00	9.00	9.00
	7.10		9.00		9.00		9.00	

Table 26 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
13	7.40		9.00		9.00		9.00	
	7.75	7.55	9.00	9.00	9.00	9.00	9.00	9.00
	7.50		9.00		9.00		9.00	
14	8.00		9.00		9.00		9.00	
	8.00	8.00	9.00	9.00	9.00	9.00	9.00	9.00
	8.00		9.00		9.00		9.00	
15	8.10		9.00		9.00		9.00	
	8.10	8.10	9.00	9.00	9.00	9.00	9.00	9.00
	8.10		9.00		9.00		9.00	
16	8.30		9.00		9.00		9.00	
	8.30	8.30	9.00	9.00	9.00	9.00	9.00	9.00
	8.30		9.00		9.00		9.00	
17	8.30		9.00		9.00		9.00	
	8.30	8.30	9.00	9.00	9.00	9.00	9.00	9.00
	8.30		9.00		9.00		9.00	
18	8.40		9.00		9.00		9.00	
	8.40	8.40	9.00	9.00	9.00	9.00	9.00	9.00
	8.40		9.00		9.00		9.00	

Table 26 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
19	8.45		9.00		9.00		9.00	
	8.45	8.45	9.00	9.00	9.00	9.00	9.00	9.00
	8.45		9.00		9.00		9.00	
20	8.50		9.00		9.00		9.00	
	8.60	8.53	9.00	9.00	9.00	9.00	9.00	9.00
	8.50		9.00		9.00		9.00	
21	8.50		9.00		9.00		9.00	
	8.60	8.53	9.00	9.00	9.00	9.00	9.00	9.00
	8.50		9.00		9.00		9.00	
22	8.50		9.00		9.00		9.00	
	8.60	8.53	9.00	9.00	9.00	9.00	9.00	9.00
	8.50		9.00		9.00		9.00	
23	8.50		9.00		9.00		9.00	
	8.60	8.53	9.00	9.00	9.00	9.00	9.00	9.00
	8.50		9.00		9.00		9.00	
24	8.50		9.00		9.00		9.00	
	8.60	8.53	9.00	9.00	9.00	9.00	9.00	9.00
	8.50		9.00		9.00		9.00	

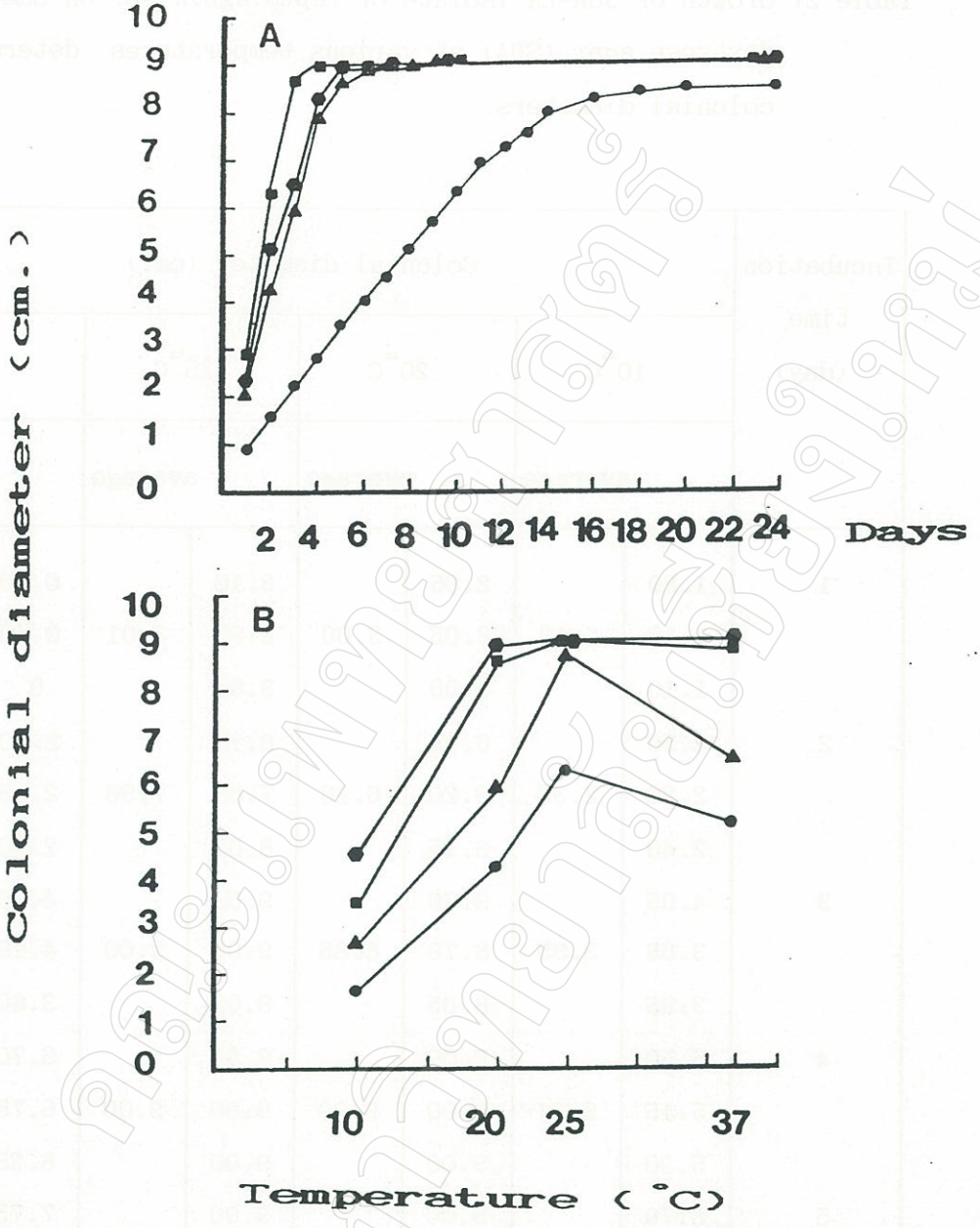

Figure 45. (A). Growth of 35C-1 isolate of *Aphanomyces sp.* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬢) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 35C-1 isolate in various temperatures determined by colonial diameters at day 2 (●), day 3 (▲), day 5 (■) and day 7 (⬢).

Table 27 Growth of 36A-LK isolate of *Leptolegnia sp.* on Sabouraud's dextrose agar (SDA) at various temperatures determined by colonial diameters.

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
1	1.20		2.95		3.10		0.80	
	1.10	1.13	3.05	3.00	2.95	3.01	0.50	0.43
	1.10		3.00		3.00		0	
2	2.50		6.15		8.10		2.70	
	2.20	2.36	6.20	6.16	7.80	7.96	2.55	2.55
	2.40		6.15		8.00		2.40	
3	4.05		9.00		9.00		4.30	
	3.85	3.95	8.70	8.85	9.00	9.00	4.10	4.00
	3.95		8.85		9.00		3.60	
4	5.50		9.00		9.00		6.70	
	5.15	5.31	9.00	9.00	9.00	9.00	6.75	6.56
	5.30		9.00		9.00		6.25	
5	6.70		9.00		9.00		7.75	
	6.50	6.61	9.00	9.00	9.00	9.00	8.70	8.11
	6.65		9.00		9.00		7.90	
6	8.80		9.00		9.00		9.00	
	8.60	7.70	9.00	9.00	9.00	9.00	9.00	9.00
	8.60		9.00		9.00		9.00	

Table 27 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
7	8.66		9.00		9.00		9.00	
	8.66	8.66	9.00	9.00	9.00	9.00	9.00	9.00
	8.66		9.00		9.00		9.00	
8	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
9	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
10	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
11	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
12	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

Table 27 (Continued)

Incubation time (day)	Colonial diameter (cm.)							
	10°C		20°C		25°C		37°C	
		average		average		average		average
13	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	
14	9.00		9.00		9.00		9.00	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00	

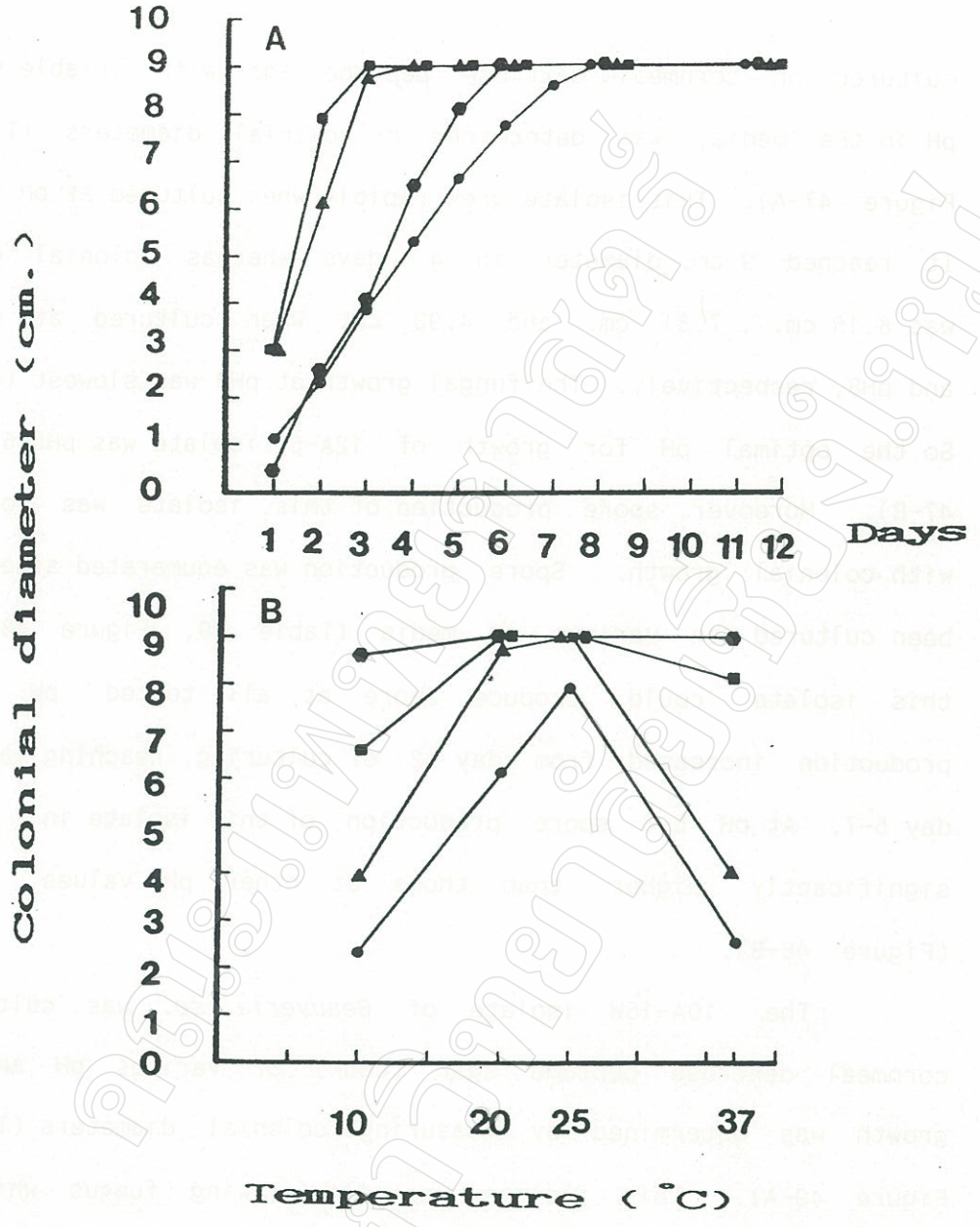

Figure 46. (A). Growth of 36A-LK isolate of *Leptolegnia sp.* in various temperatures; 10°C (●), 20°C (▲), 25°C (■) and 37°C (⬢) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 36A-LK isolate in various temperatures determined by colonial diameters at day 2 (●), day 3 (▲), day 5 (■) and day 7 (⬢).

cultured on cornmeal dextrose peptone agar with variable value of pH in the media, was determined by colonial diameters (Table 28, Figure 47-A). This isolate grew rapidly when cultured at pH 5 and 6. It reached 9 cm. diameter in 4 days whereas colonial diameter was 8.15 cm. , 7.31 cm. and 4.93 cm. when cultured at pH4, pH7 and pH8, respectively. The fungal growth at pH8 was slowest ($p<0.01$). So the optimal pH for growth of 12A-6 isolate was pH5-6 (Figure 47-B). Moreover, spore production of this isolate was correlated with colonial growth. Spore production was enumerated after it had been cultured on various pH media (Table 29, Figure 48-A) and this isolate could produce spore at all tested pH. Spore production increased from day 2 of culturing, reaching maximum on day 5-7. At pH 5-6 spore production of this isolate in 1 week was significantly higher than those at other pH values ($p<0.05$) (Figure 48-B).

The 10A-15W isolate of *Beauveria sp.* was cultured on cornmeal dextrose peptone agar (CMDP) of various pH and fungal growth was determined by measuring colonial diameters (Table 30, Figure 49-A). This isolate was slow-growing fungus which grew quite well at pH 8, 7 and 6 ($p<0.01$). Whilst at lower pH (pH 5), this isolate grew slowly and could not grow at all on pH4 medium. So, the optimal pH for growth of this isolate was ranging from pH 6 to pH 8 (Figure 49-B). This fungus could produce spores at all pH excepted pH 4 (Table 31, Figure 50-A). Most spore production

Table 28 Growth of 12A-6 isolate of *Trichoderma viride* on cornmeal dextrose peptone agar (CMDP) of various pH determined by colonial diameters at room temperature.

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
1	2.20		1.00		1.75		1.80		1.30		0.95	
	2.20	2.23	1.05	1.05	1.75	1.78	1.70	1.73	1.10	1.16	1.00	0.98
	2.30		1.10		1.85		1.70		1.10		1.00	
2	5.35		2.80		4.30		4.25		2.65		1.80	
	5.40	5.38	2.70	2.83	4.45	4.38	4.10	4.13	2.60	2.58	1.80	1.85
	5.40		3.00		4.40		4.05		2.50		1.95	
3	8.50		5.00		6.95		6.80		4.55		3.00	
	8.40	8.43	4.75	5.01	6.90	6.91	6.75	6.73	4.60	4.50	3.00	3.00
	8.40		5.30		6.90		6.65		4.35		3.00	
4	9.00		8.20		9.00		9.00		7.15		4.95	
	9.00	9.00	7.95	8.15	9.00	9.00	9.00	9.00	7.50	7.31	4.90	4.93
	9.00		8.30		9.00		9.00		7.30		4.95	
5	9.00		9.00		9.00		9.00		8.50		6.30	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	8.50	8.50	6.00	5.93
	9.00		9.00		9.00		9.00		8.50		5.50	
6	9.00		9.00		9.00		9.00		8.50		6.30	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	8.50	8.50	6.00	5.93
	9.00		9.00		9.00		9.00		8.50		5.50	
7	9.00		9.00		9.00		9.00		8.50		6.30	
	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00	8.50	8.50	6.00	5.93
	9.00		9.00		9.00		9.00		8.50		5.50	

Note: * Cornmeal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control (pH6±0.2)

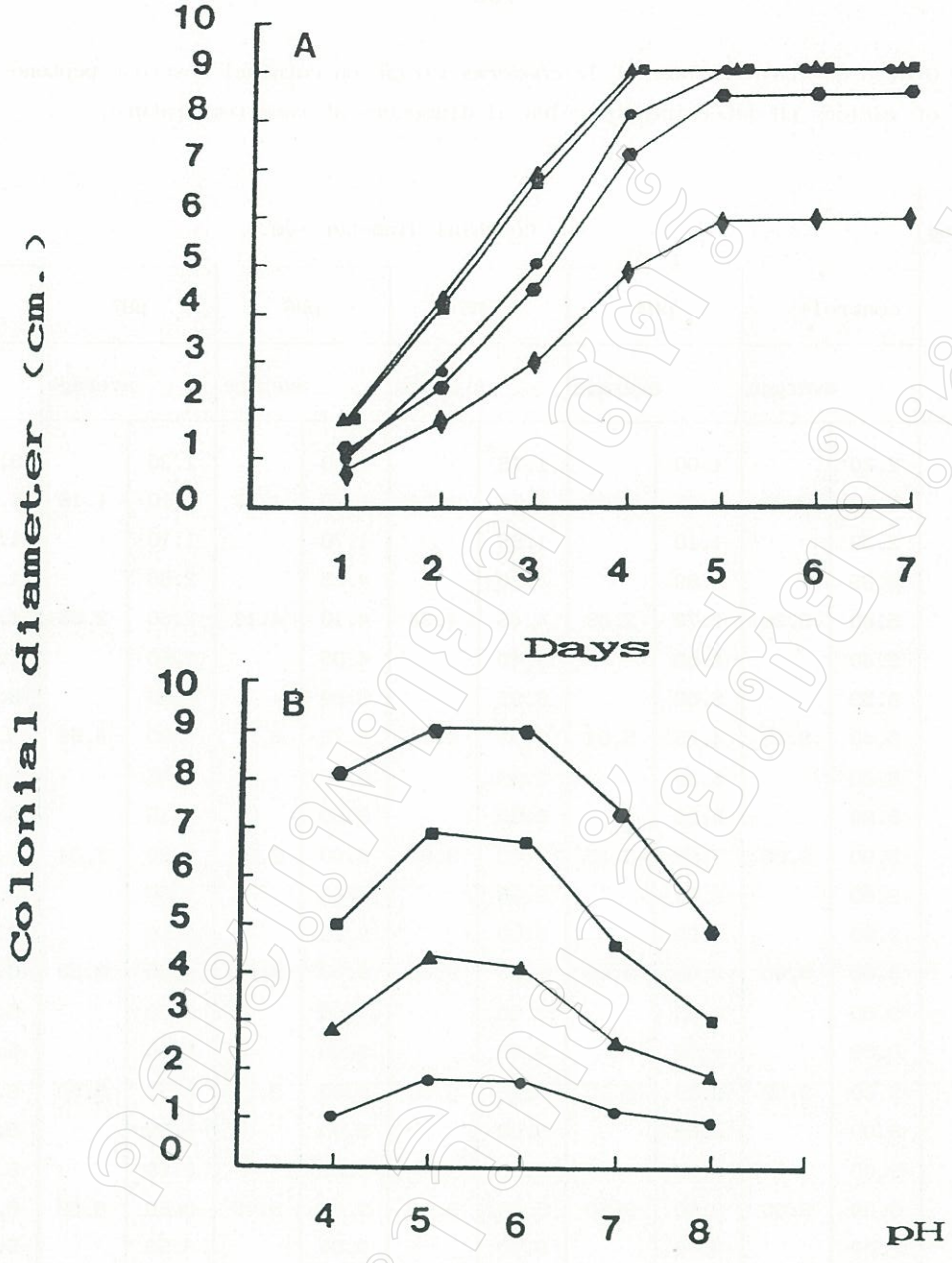

Figure 47. (A). Growth of 12A-6 isolate of *Trichoderma viride* on various pH media ; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH8 (♦) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 12A-6 isolate on various pH media determined on day 1 (●), day 2 (▲), day 3 (■), and day 4 (⬢) of cultures by colonial diameters.

Table 29 Spore production of 12A-6 isolate of *Trichoderma viride* on different pH media after various incubation times at room temperature.

Incubation time (day)	Spore enumeration (Spores/ml)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
2	8.00×10^{5}		3.60×10^{5}		1.28×10^{6}		1.05×10^{6}		9.62×10^{5}		7.25×10^{5}	
	6.50×10^{5}	7.25×10^{5}	5.15×10^{5}	4.37×10^{5}	1.04×10^{6}	1.16×10^{6}	8.05×10^{5}	9.30×10^{5}	8.35×10^{6}	8.98×10^{5}	5.17×10^{5}	6.21×10^{5}
3	2.69×10^{7}		1.29×10^{7}		2.50×10^{7}		2.58×10^{7}		6.05×10^{6}		2.26×10^{6}	
	2.97×10^{7}	2.83×10^{7}	1.44×10^{7}	1.36×10^{7}	2.23×10^{7}	2.36×10^{7}	3.97×10^{7}	3.27×10^{7}	6.90×10^{6}	6.47×10^{6}	1.17×10^{6}	1.71×10^{6}
5	3.37×10^{6}		9.90×10^{7}		1.14×10^{8}		1.49×10^{8}		1.28×10^{8}		7.10×10^{7}	
	3.51×10^{6}	3.44×10^{6}	1.26×10^{8}	1.12×10^{8}	1.25×10^{8}	1.19×10^{8}	1.57×10^{8}	1.53×10^{8}	8.30×10^{7}	1.05×10^{8}	7.30×10^{7}	7.20×10^{7}
7	4.89×10^{6}		1.56×10^{8}		2.27×10^{8}		2.42×10^{8}		1.81×10^{8}		1.41×10^{8}	
	4.89×10^{6}	4.89×10^{6}	1.45×10^{8}	1.50×10^{8}	2.31×10^{8}	2.29×10^{8}	2.23×10^{8}	2.32×10^{8}	1.85×10^{8}	1.83×10^{8}	1.05×10^{8}	1.23×10^{8}
14	9.20×10^{7}		9.12×10^{7}		2.13×10^{8}		2.17×10^{8}		1.09×10^{8}		7.45×10^{7}	
	8.80×10^{7}	9.00×10^{7}	8.88×10^{7}	9.00×10^{7}	1.87×10^{8}	2.00×10^{8}	3.23×10^{8}	2.70×10^{8}	9.10×10^{7}	1.00×10^{8}	7.15×10^{7}	7.30×10^{7}

Note: * Cornmeal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control.

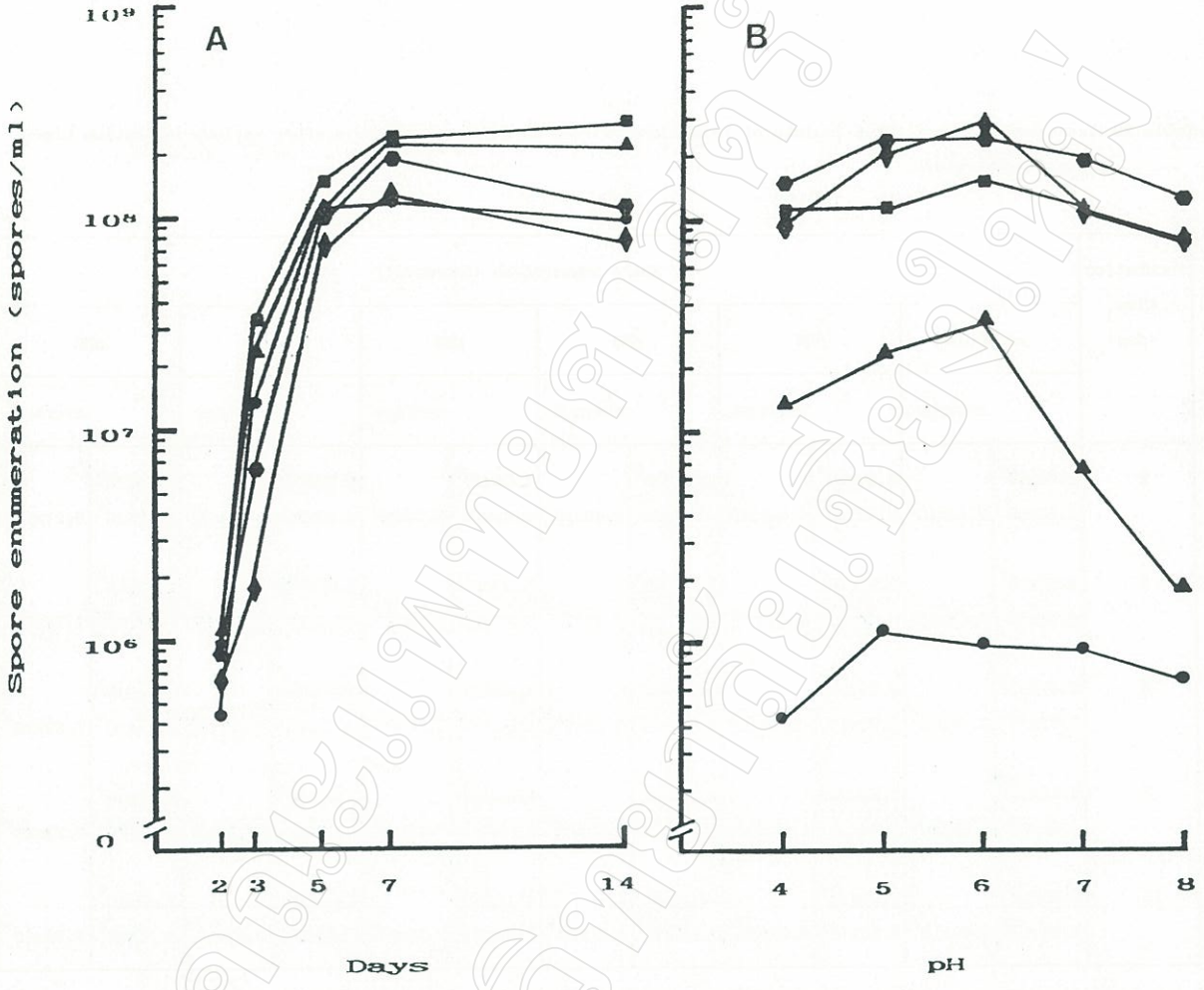

Figure 48. (A). Spore production of 12A-6 isolate of *Trichoderma viride* cultured on various pH media ; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH 8 (⧫). Results represent the average of triplicate cultures.

(B). Spore production of 12A-6 isolate cultured for 2 days (●), 3 days (▲), 5 days (■), 7 days (⬢) and 14 days (⧫) on various pH media.

Table 30 Growth of 10A-15W isolate of *Beauveria sp.* on conmeal dextrose peptone agar (CMDP) of various pH determined by colonial diameters at room temperature.

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
1	0		0		0		0		0		0	
	0	0	0	0	0	0	0	0	0	0	0	0
	0		0		0		0		0		0	
2	0.85		0		0		0.40		0.55		0.80	
	0.75	0.81	0	0	0	0	0.40	0.41	0.80	0.65	0.70	0.70
	0.85		0		0		0.45		0.60		0.60	
3	1.10		0		0		0.45		0.85		1.00	
	1.05	1.10	0	0	0	0	0.55	0.55	1.05	0.95	0.95	0.96
	1.15		0		0		0.65		0.95		0.95	
4	1.30		0		0		0.65		1.05		1.30	
	1.25	1.28	0	0	0	0	0.70	0.75	1.40	1.20	1.20	1.20
	1.30		0		0		0.90		1.15		1.10	
5	1.85		0		0		1.15		1.60		1.85	
	1.90	1.83	0	0	0	0	1.20	1.21	1.90	1.70	1.70	1.71
	1.75		0		0		1.30		1.60		1.60	
6	2.20		0		0		1.20		1.85		2.05	
	2.10	2.08	0	0	0	0	1.40	1.40	2.05	1.88	1.90	1.91
	1.95		0		0		1.60		1.75		1.80	

Table 30 (Continued)

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
7	2.60		0		0.10		1.60		2.15		2.30	
	2.50	2.50	0	0	0.10	0.13	1.70	1.73	2.40	2.26	2.25	2.18
	2.40		0		0.20		1.90		2.25		2.00	
8	3.05		0		0.10		1.90		2.50		2.70	
	3.10	3.01	0	0	0.10	0.13	2.10	2.05	2.70	2.55	2.60	2.56
	2.90		0		0.20		2.15		2.45		2.40	
9	3.40		0		0.35		2.15		2.20		3.00	
	3.30	3.30	0	0	0.20	0.33	2.30	2.26	2.95	2.65	2.80	2.86
	3.20		0		0.45		2.35		2.80		2.80	
10	3.80		0		0.65		2.50		3.05		3.30	
	3.80	3.76	0	0	0.65	0.60	2.70	2.55	3.30	3.16	3.20	3.23
	3.70		0		0.50		2.45		3.15		3.20	
11	4.20		0		0.85		2.80		3.35		3.60	
	4.30	4.23	0	0	0.70	0.81	3.00	2.90	3.65	3.50	3.55	3.55
	4.20		0		0.90		2.90		3.50		3.50	
12	4.20		0		1.15		3.00		3.45		4.00	
	4.40	4.30	0	0	0.95	1.08	3.20	2.98	3.80	3.63	3.80	3.86
	4.30		0		1.15		2.75		3.65		3.80	
13	5.10		0		1.50		3.35		3.75		4.30	
	5.00	5.06	0	0	1.30	1.45	3.35	3.23	4.50	4.08	4.20	4.23
	5.10		0		1.55		3.00		4.00		4.20	

Table 30 (Continued)

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
14	5.30		0		1.85		3.50		4.10		4.60	
	5.20	5.23	0	0	1.60	1.75	3.50	3.40	4.10	4.10	4.60	4.60
	5.20		0		1.80		3.20		4.10		4.60	
15	5.50		0		2.00		3.85		4.15		4.80	
	5.50	5.50	0	0	1.80	1.95	3.85	3.66	4.20	4.31	4.80	4.80
	5.50		0		2.05		3.30		4.60		4.80	
16	6.00		0		2.35		4.20		4.70		5.15	
	6.70	6.46	0	0	2.05	2.23	-**	4.20	4.90	4.81	5.05	5.08
	6.70		0		2.30		-		4.85		5.05	
17	6.40		0		2.60		4.50		4.80		5.30	
	6.30	6.33	0	0	2.35	2.50	-	4.50	5.00	4.96	5.40	5.36
	6.30		0		2.55		-		5.10		5.40	
18	6.50		0		2.80		4.70		5.20		5.70	
	6.50	6.50	0	0	2.50	2.70	-	4.70	5.10	5.21	5.65	5.66
	6.50		0		2.80		-		5.35		5.65	
19	6.80		0		3.10		4.90		5.30		5.60	
	6.80	6.80	0	0	2.90	3.00	-	4.90	5.30	5.38	5.60	5.73
	6.80		0		3.00		-		5.55		5.80	

Note: * Cornmeal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control.

** Colonial diameter could not determine because of contamination.

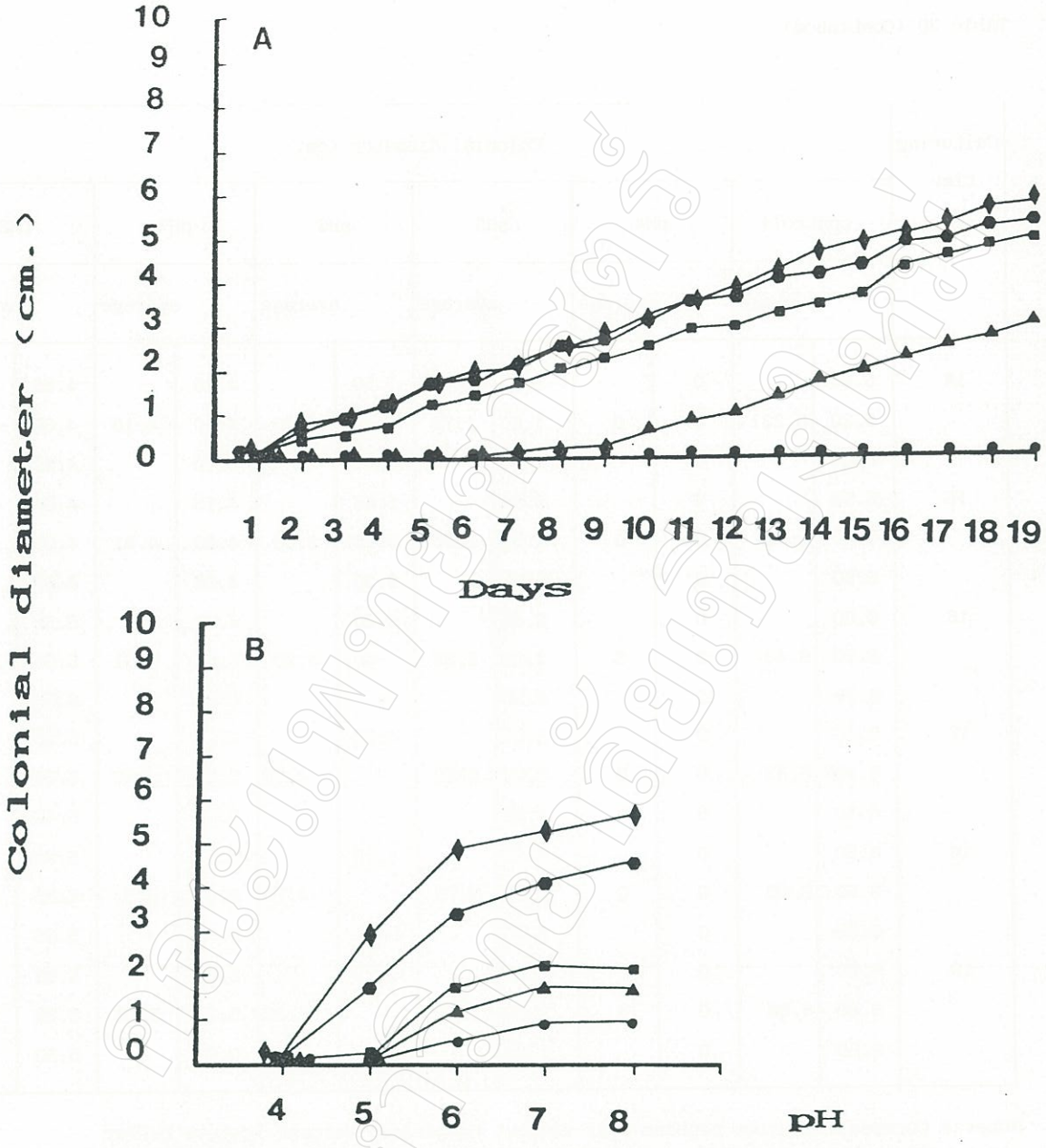

Figure 49. (A). Growth of 10A-15W isolate of *Beauveria sp.* on various pH media ; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH8 (♦) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 10A-15W isolate on various pH media determined on day 3 (●), day 5 (▲), day 7 (■), day 14 (⬢) and day 19 (♦) of cultures by colonial diameters.

Table 31 Spore production of 10A-15W isolate of *Beauveria sp.* on different pH media after various incubation times at room temperature.

Incubation time (day)	Spore enumeration (Spores/ml)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
3	4.00×10^{5}		0		5.50×10^{5}		5.40×10^{5}		6.10×10^{5}		4.30×10^{5}	
	4.20×10^{5}	4.10×10^{5}	0	0	4.10×10^{5}	4.80×10^{5}	4.90×10^{5}	5.15×10^{5}	5.00×10^{5}	5.55×10^{5}	4.10×10^{5}	4.20×10^{5}
5	1.72×10^{6}		0		2.20×10^{5}		7.80×10^{5}		4.81×10^{6}		3.88×10^{6}	
	2.21×10^{6}	1.96×10^{6}	0	0	3.90×10^{5}	3.05×10^{5}	6.50×10^{5}	7.15×10^{5}	4.70×10^{6}	4.75×10^{6}	5.43×10^{6}	4.65×10^{6}
7	3.00×10^{6}		0		7.00×10^{5}		1.90×10^{6}		1.28×10^{7}		2.46×10^{7}	
	3.62×10^{6}	3.31×10^{6}	0	0	6.20×10^{5}	6.60×10^{5}	2.04×10^{6}	1.97×10^{6}	1.52×10^{7}	1.40×10^{7}	2.29×10^{7}	2.37×10^{7}
14	8.90×10^{6}		0		1.70×10^{7}		1.26×10^{7}		1.25×10^{7}		5.55×10^{6}	
	1.24×10^{7}	1.06×10^{7}	0	0	1.48×10^{7}	1.59×10^{7}	1.35×10^{7}	1.30×10^{7}	1.16×10^{7}	1.20×10^{7}	5.25×10^{6}	5.40×10^{6}
20	9.00×10^{6}		0		7.70×10^{6}		3.30×10^{6}		5.30×10^{6}		4.50×10^{6}	
	9.95×10^{6}	9.47×10^{6}	0	0	5.25×10^{6}	6.47×10^{6}	3.55×10^{6}	3.42×10^{6}	4.20×10^{6}	4.75×10^{6}	7.05×10^{6}	5.77×10^{6}
24	9.00×10^{6}		0		1.50×10^{7}		5.00×10^{6}		4.20×10^{6}		1.10×10^{7}	
	8.55×10^{6}	8.77×10^{6}	0	0	1.85×10^{7}	1.67×10^{7}	4.85×10^{6}	4.92×10^{6}	4.70×10^{6}	4.45×10^{6}	1.52×10^{7}	1.31×10^{7}

Note: * Cornmal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control.

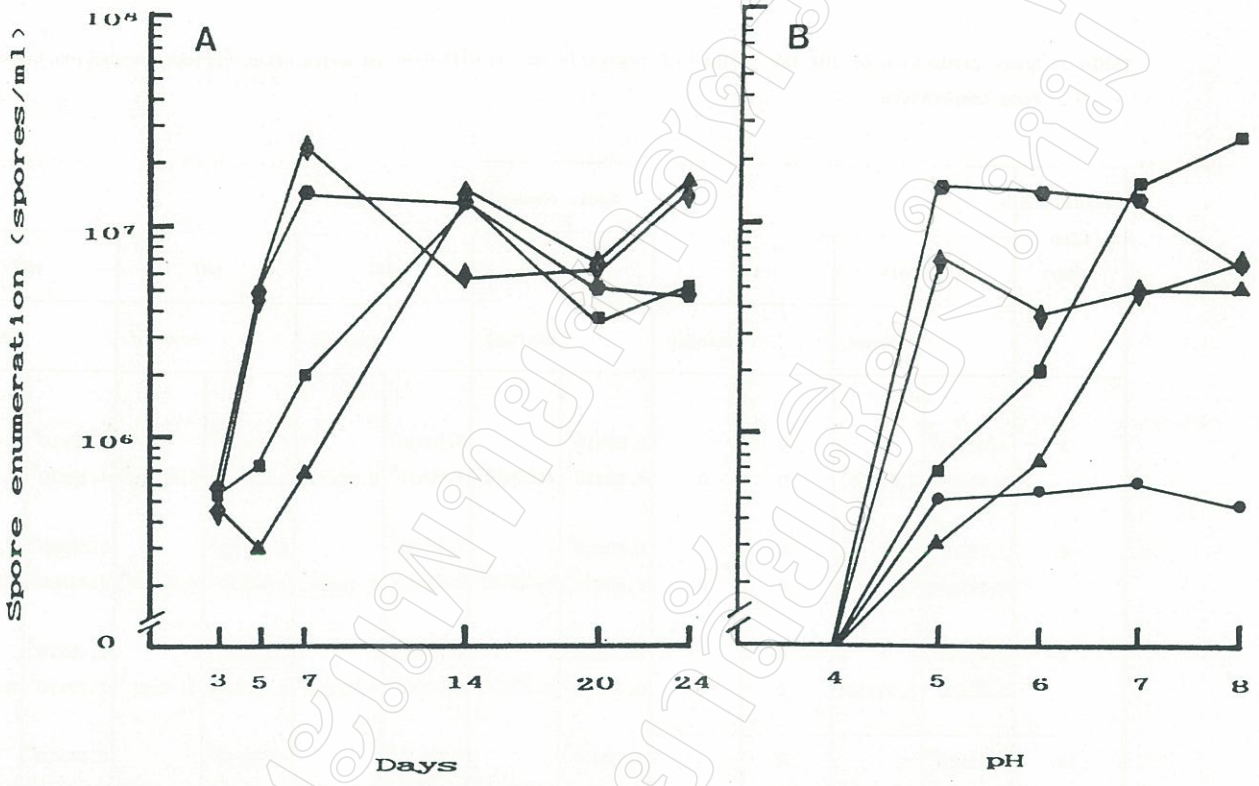

Figure 50. (A). Spore production of 10A-15w isolate of *Beauveria sp.* cultured on various pH media; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬣) and pH 8 (⧫). Results represent the average of triplicate cultures.
(B). Spore production of 10A-15W isolate cultured for 3 days (●), 5 days (▲), 7 days (■), 14 days (⬣) and 20 days (⧫) on various pH media.

was obtained at pH7 and pH8. For one week cultures, at pH7 and pH8, the spore production was significantly higher than at other pHs. While at lower pH (pH5 or 6), the spore enumeration curve slowly increased to reach maximum after this fungus had been allowed to grow for 2 weeks (Figure 50-B).

The 29B-5W isolate of *Aspergillus niveus* had the ability to grow in wide range of pH (Table 32, Figure 51-A). The colonial diameters on cornmeal dextrose peptone agar at pH5, 6, 7 and 8 after 24 days of incubation at room temperature reached 7.15, 7.53, 7.20, and 7.30 cm., respectively. At pH4, the growth was slower ($p<0.05$). However, within 7 days of incubation, at pH5, the growth was more abundant than those on pH4, 6, 7, and 8 (Figure 51-B). Although the ascospore production at pH4 was detected first, at day 14 of culture, the culture of 30 day old at pH5 yielded the highest ascospore production (Table 33, Figure 52-A). Furthermore, ascospore production on pH5 and pH6 media after 24 days of incubation were significantly higer ($p<0.05$) than those on other pH media (Figure 52-B).

The 35C-1 isolate of *Aphanomyces sp.* had the ability to grow in wide range of pH (Table 34, Figure 53-A). The colonial diameters on Sabouraud's dextrose agar at pH5, 6, 7 and 8 after 5 days of incubation at room temperature reached 9.00 cm. Whereas at pH4, the growth was too slow ($p<0.01$). Thus, the optimal pH for growth of this isolate ranged from pH5 to pH8 (Figure 53-B).

The mosquito larvae killing fungus, 36A-LK isolate of

Table 32 Growth of 29B-5W isolate of *Aspergillus niveus* on cornmeal dextrose peptone agar (CMDP) of various pH determined by colonial diameters at room temperature.

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
1	0		0		0		0		0		0	
	0	0	0	0	0	0	0	0	0	0	0	0
	0		0		0		0		0		0	
2	0.55		0		0.35		0.30		0.20		0	
	0.35	0.50	0	0	0.35	0.43	0.35	0.30	0.30	0.20	0.10	0.06
	0.60		0		0.60		0.25		0.10		0.10	
3	1.70		0.10		0.85		0.70		0.60		0.30	
	1.35	1.58	0.10	0.10	1.00	0.95	0.70	0.66	0.65	0.56	0.75	0.53
	1.70		0.10		1.00		0.60		0.45		0.55	
4	2.40		0.25		1.25		0.90		0.85		0.65	
	2.10	2.31	0.10	0.16	1.20	1.23	1.00	0.90	0.95	0.85	1.05	0.86
	2.45		0.15		1.25		0.80		0.75		0.90	
5	3.20		0.45		1.50		1.15		1.10		0.95	
	2.85	3.08	0.20	0.30	1.60	1.53	1.25	1.16	1.30	1.13	1.30	1.13
	3.20		0.25		1.50		1.10		1.00		1.15	
6	3.90		0.65		2.00		1.50		1.45		1.25	
	3.65	3.86	0.50	0.51	2.00	1.93	1.55	1.48	1.50	1.41	1.70	1.50
	4.05		0.40		1.80		1.40		1.30		1.55	

Table 32 (Continued)

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
7	4.50		0.90		2.20		1.75		1.75		1.60	
	4.35	4.53	0.65	0.71	2.05	2.11	1.80	1.76	1.85	1.75	1.90	1.75
	4.75		0.60		2.10		1.75		1.65		1.75	
8	5.30		1.30		2.50		2.10		2.05		1.90	
	5.20	5.20	1.05	1.08	2.55	2.50	2.15	2.10	2.10	2.03	2.25	2.10
	5.10		0.90		2.45		2.05		1.95		2.15	
9	6.00		1.60		2.90		2.45		2.40		2.30	
	6.10	6.23	1.35	1.43	3.00	2.88	2.55	2.48	2.45	2.41	2.65	2.50
	6.60		1.35		2.75		2.45		2.40		2.55	
10	6.50		1.85		3.15		2.60		2.65		2.55	
	6.65	6.71	1.55	1.66	3.30	3.18	2.70	2.65	2.75	2.66	2.85	2.71
	7.00		1.60		3.10		2.65		2.60		2.75	
11	7.30		2.25		3.35		3.05		3.15		2.85	
	7.30	7.41	1.85	2.03	3.55	3.43	3.05	3.03	3.15	3.06	3.20	3.06
	7.65		2.00		3.40		3.00		2.90		3.15	
12	7.70		2.60		3.85		3.35		3.50		3.30	
	7.80	7.80	2.15	2.38	4.00	3.90	3.50	3.43	3.40	3.41	3.60	3.46
	7.90		2.40		3.85		3.45		3.35		3.50	

Table 32 (Continued)

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
13	8.30		2.85		4.00		3.60		3.70		3.55	
	8.40	8.36	2.35	2.60	4.05	4.03	3.75	3.68	3.70	3.66	3.85	3.75
	8.40		2.60		4.05		3.70		3.60		3.85	
14	8.40		3.15		4.30		4.00		4.00		3.95	
	8.50	8.43	2.55	2.90	4.50	4.43	4.00	4.03	4.10	4.03	4.30	4.13
	8.40		3.00		4.50		4.10		4.00		4.15	
15	8.40		3.40		4.75		4.50		4.25		4.30	
	8.50	8.43	2.85	3.18	4.85	4.80	4.65	4.60	4.50	4.38	4.70	4.55
	8.40		3.30		4.80		4.65		4.40		4.65	
16	8.40		3.60		4.80		4.70		4.90		4.50	
	8.50	8.46	3.05	3.38	5.25	5.05	4.80	4.78	4.90	4.83	4.80	4.75
	8.50		3.50		5.10		4.85		4.70		4.90	
17	8.50		3.85		5.30		5.05		5.10		4.90	
	8.50	8.50	3.25	3.60	5.60	5.46	5.10	5.11	5.10	5.10	5.20	5.10
	8.50		3.70		5.50		5.20		5.10		5.20	
18	8.70		4.20		5.75		5.50		5.60		5.40	
	8.70	8.70	3.60	3.96	6.05	6.00	5.60	5.53	5.60	5.56	5.70	5.60
	8.70		4.10		6.20		5.50		5.50		5.70	

Table 32 (Continued)

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
21	8.70		5.15		6.70		6.40		6.60		6.40	
	8.70	8.70	4.30	4.73	7.05	6.96	6.60	6.53	6.60	6.56	6.90	6.71
	8.70		4.75		7.15		6.60		6.50		6.85	
24	8.80		6.00		6.80		7.20		7.20		7.00	
	8.90	8.83	5.20	5.61	7.15	7.15	7.70	7.53	7.20	7.20	7.50	7.33
	8.80		5.65		7.50		7.70		7.20		7.50	

Note: * Conmeal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control.

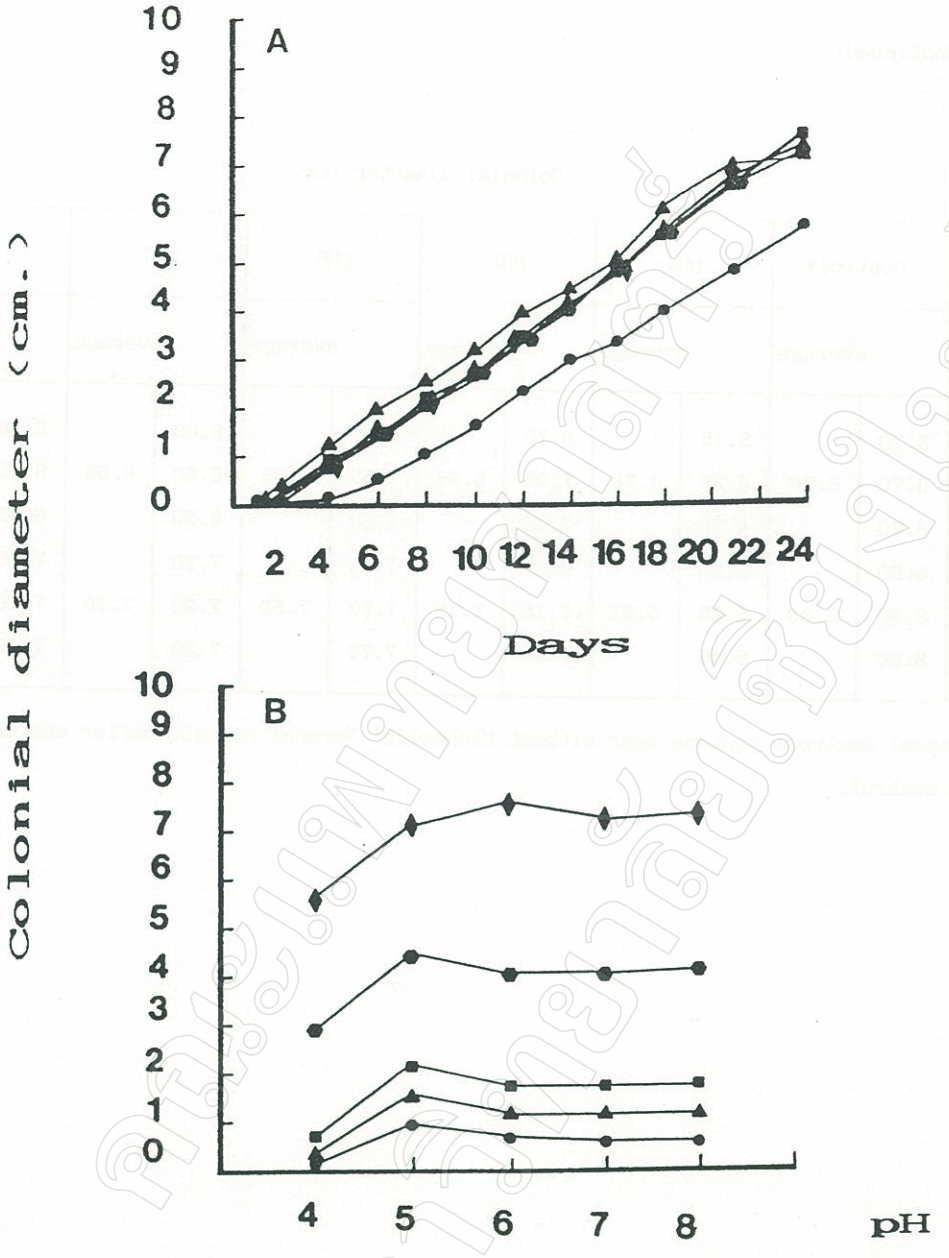

Figure 51. (A). Growth of 29B-5W isolate of *Aspergillus niveus* on various pH media : pH4 (●), pH5 (▲), pH6 (■), pH7 (⬣) and pH8 (⧫) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 29B-5W isolate on various pH media determined on day 3 (●), day 5 (▲), day 7 (■), day 14 (⬣) and day 24 (⧫) of cultures by colonial diameters.

Table 33 Ascospore production of 23B-5W isolate of *Aspergillus niveus* on different pH media after various incubation times at room temperature.

Incubation time (day)	Spore enumeration (Spores/ml)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
3	0		0		0		0		0		0	
	0	0	0	0	0	0	0	0	0	0	0	0
5	0		0		0		0		0		0	
	0	0	0	0	0	0	0	0	0	0	0	0
7	4.50×10^{4}		0		0		0		0		0	
	5.25×10^{4}	4.87×10^{4}	0	0	0	0	0	0	0	0	0	0
14	4.72×10^{6}		1.50×10^{4}		0		0		0		0	
	4.95×10^{6}	4.83×10^{6}	5.00×10^{3}	1.00×10^{4}	0	0	0	0	0	0	0	0
20	5.45×10^{6}		9.50×10^{4}		6.25×10^{4}		1.35×10^{5}		0		0	
	4.87×10^{6}	5.16×10^{6}	6.00×10^{4}	7.75×10^{4}	5.00×10^{4}	5.62×10^{4}	6.75×10^{4}	1.01×10^{5}	0	0	0	0
24	4.09×10^{6}		1.42×10^{5}		3.44×10^{6}		2.91×10^{6}		2.00×10^{4}		2.50×10^{3}	
	5.00×10^{6}	4.54×10^{6}	1.87×10^{5}	1.65×10^{5}	3.15×10^{6}	3.29×10^{6}	2.80×10^{6}	2.85×10^{6}	1.25×10^{4}	1.62×10^{4}	1.00×10^{4}	6.25×10^{3}
30	2.79×10^{7}		4.10×10^{5}		1.23×10^{7}		5.30×10^{6}		2.05×10^{5}		2.00×10^{4}	
	3.01×10^{7}	2.90×10^{7}	5.20×10^{5}	4.65×10^{5}	1.93×10^{7}	1.58×10^{7}	5.01×10^{6}	5.15×10^{6}	2.37×10^{5}	2.21×10^{5}	3.00×10^{4}	2.50×10^{4}

Note: * Cornmeal dextrose peptone agar without Michaelis' Veronal Acetate buffer was used as control.

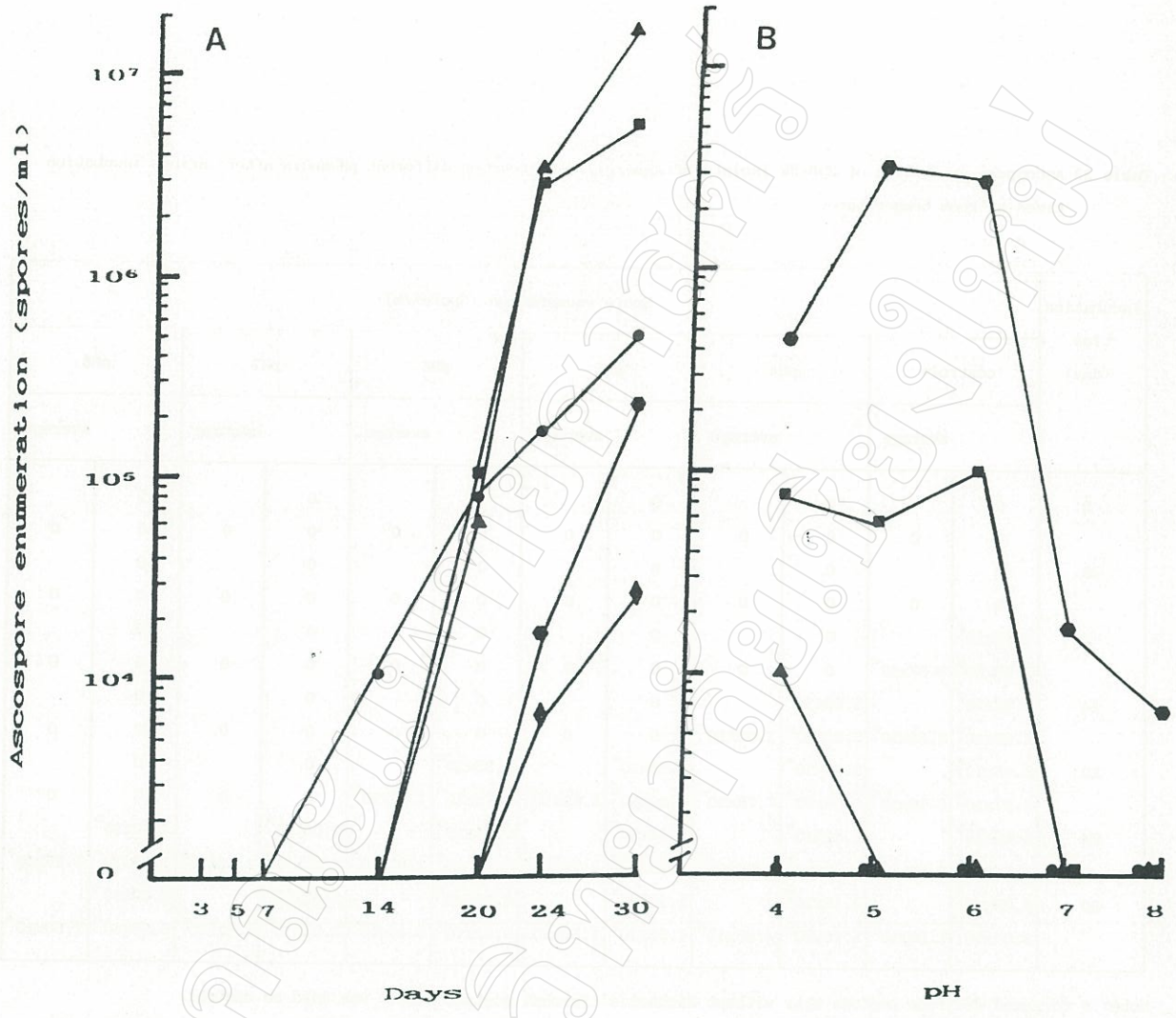

Figure 52. (A). Ascospore production of 29B-5W isolate of *Aspergillus niveus* cultured on various pH media; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH8 (♦). Results represent the average of triplicate cultures.

(B). Ascospore production of 29B-5W isolate cultured for 7 days (●), 14 days (▲), 20 days (■), and 24 days (⬢) on various pH media.

Table 34 Growth of 35C-1 isolate of *Aphanomyces sp.* on Sabouraud's dextrose agar (SDA) of various pH determined by colonial diameters at room temperature.

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
1	2.70		0		2.20		2.70		2.95		2.80	
	2.75	2.73	0	0	2.15	2.18	2.70	2.70	2.90	2.88	2.75	2.80
	2.75		0		2.20		2.70		2.80		2.85	
2	5.60		0		5.00		5.70		5.80		5.70	
	5.70	5.68	0.60	0.20	5.00	5.00	5.70	5.70	5.80	5.78	5.70	5.70
	5.75		0		5.00		5.70		5.75		5.70	
3	7.95		0		7.30		8.20		8.30		8.25	
	8.20	8.11	0.80	0.26	7.25	7.28	8.20	8.21	8.30	8.20	8.20	8.30
	8.20		0		7.30		8.25		8.00		8.45	
4	9.00		0		8.50		9.00		9.00		9.00	
	9.00	9.00	1.20	0.40	8.60	8.63	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		0		8.80		9.00		9.00		9.00	
5	9.00		1.70		9.00		9.00		9.00		9.00	
	9.00	9.00	1.70	1.70	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		1.70		9.00		9.00		9.00		9.00	
6	9.00		2.35		9.00		9.00		9.00		9.00	
	9.00	9.00	2.35	2.35	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		2.35		9.00		9.00		9.00		9.00	
14	9.00		8.50		9.00		9.00		9.00		9.00	
	9.00	9.00	8.50	8.50	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		8.50		9.00		9.00		9.00		9.00	

Note: * Sabouraud's dextrose agar without Michaelis' Veronal Acetate buffer was used as control.

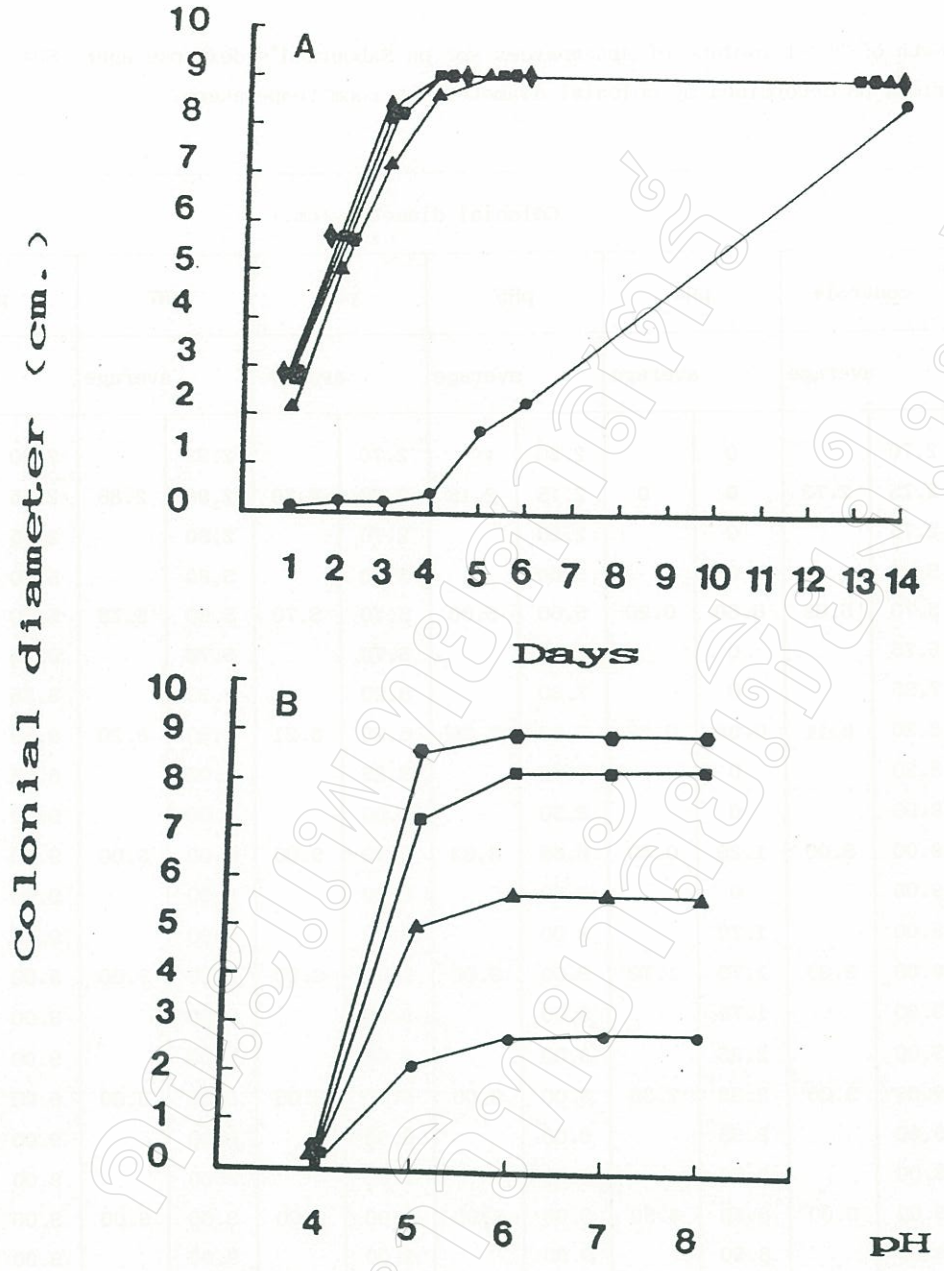

Figure 53. (A). Growth of 35C-1 isolate of *Aphanomyces sp.* on various pH media ; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH8 (♦) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 35C-1 isolate on various pH media determined on day 1 (●), day 2 (▲), day 3 (■), and day 4 (⬢) of cultures by colonial diameters.

Leptolegnia sp. could grow well on Sabouraud's dextrose agar with pH6-8 ($p<0.01$)(Table 35, Figure 54-A). Colonial diameters of fungal growth were detected and they reached 9.00 cm. on day 4 of culturing whereas it took 14 days when growing on pH5 media and this fungal isolate slightly grew on pH4 media. Colonial diameter was 5.66 cm. eventhough it was allowed to grow for 14 days. Thus, the optimal pH for growth of 36A-LK isolate of *Leptolegnia sp.* ranged from pH6 to pH8 (Figure 54-B).

2.3 Nutritional requirements

2.3.1 Carbon assimilation test

Carbon assimilation of each isolate was tested with various carbon sources (Table 36). *Candida albicans, Candida krusei* and *Aspergillus flavus* were used as controls in this test. *Candida albicans* could assimilate glucose, maltose, D-galactose and saccharose, but not lactose whereas *C.krusei* could assimilate only glucose. Hence, *Aspergillus flavus* which could assimilate all sugars in this test, was used as positive control. All isolates of mosquito larvae killing fungi could assimilate glucose and 3 isolates, 12A-6 (*Trichoderma viride*), 10A-15W (*Beauveria sp.*) and 29B-5W (*Aspergillus niveus*) could assimilate all sugars; glucose, maltose, D-galactose, lactose and saccharose, whereas

Table 35 Growth of 36A-LK isolate of *Leptolegnia sp.* on Sabouraud's dextrose agar (SDA) of various pH determined by colonial diameters at room temperature.

Culturing time (day)	Colonial diameter (cm.)											
	control*		pH4		pH5		pH6		pH7		pH8	
		average		average		average		average		average		average
1	3.50		0		0.60		3.20		3.70		3.20	
	3.40	3.46	0	0	0.80	0.73	2.95	2.96	3.55	3.58	3.20	3.23
	3.50		0		0.80		2.75		3.50		3.30	
2	7.50		0		1.00		6.60		7.80		7.90	
	7.50	7.56	0	0	1.10	1.06	6.50	6.48	7.90	7.76	7.75	7.83
	7.70		0		1.10		6.35		7.60		7.85	
3	9.00		0		1.85		8.90		9.00		9.00	
	9.00	9.00	0	0	2.00	1.91	9.00	8.96	9.00	9.00	9.00	9.00
	9.00		0		1.90		9.00		9.00		9.00	
4	9.00		0		2.75		9.00		9.00		9.00	
	9.00	9.00	0	0.28	3.00	2.83	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		0.85		2.75		9.00		9.00		9.00	
5	9.00		0		4.85		9.00		9.00		9.00	
	9.00	9.00	0	0.46	5.10	4.86	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		1.40		4.65		9.00		9.00		9.00	
6	9.00		0		6.80		9.00		9.00		9.00	
	9.00	9.00	0.60	0.93	7.45	7.10	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		2.20		7.05		9.00		9.00		9.00	
14	9.00		0		9.00		9.00		9.00		9.00	
	9.00	9.00	8.00	5.66	9.00	9.00	9.00	9.00	9.00	9.00	9.00	9.00
	9.00		9.00		9.00		9.00		9.00		9.00	

Note: * Sabouraud's dextrose agar without Michaelis' Veronal Acetate buffer was used as control.

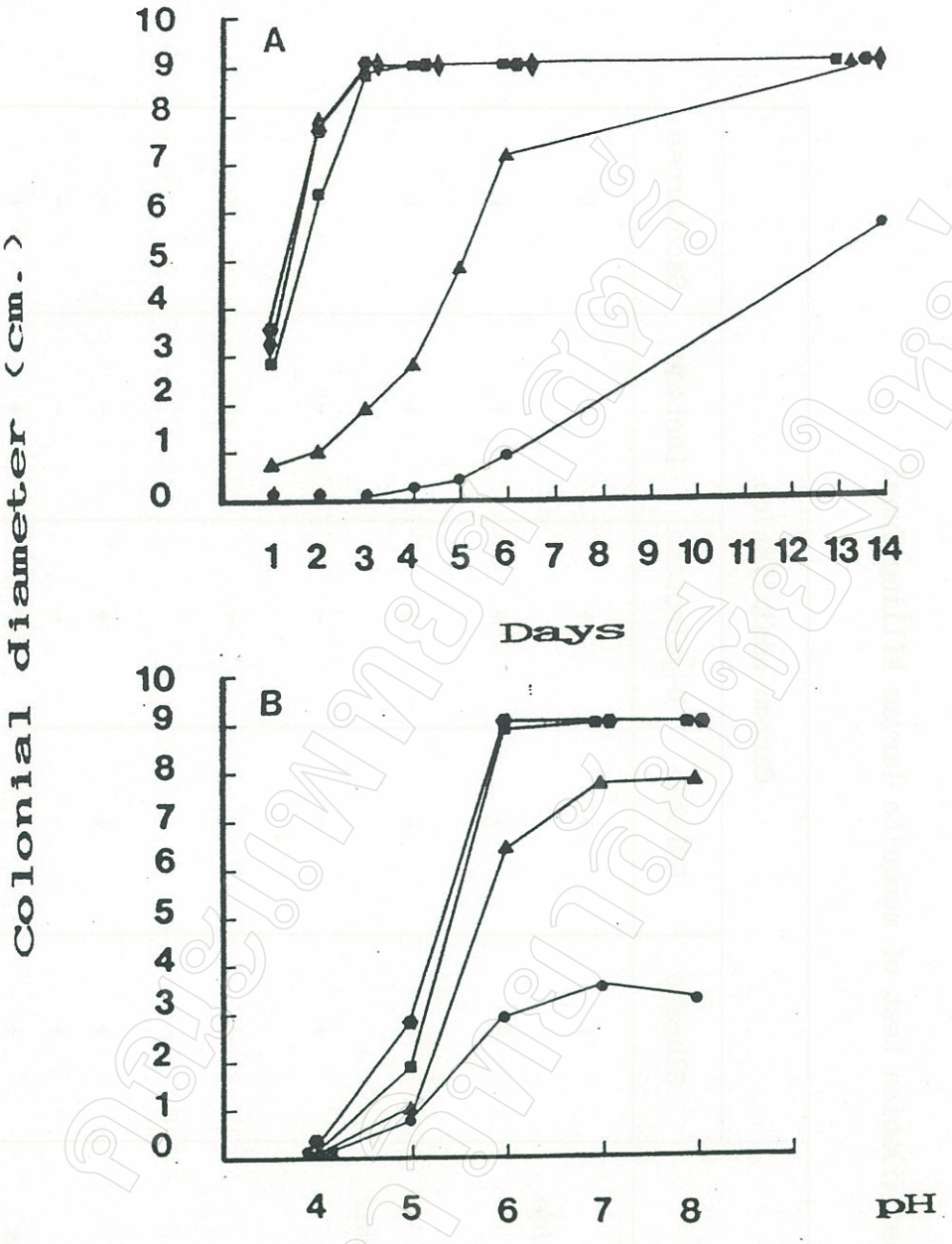

Figure 54. (A). Growth of 36A-LK isolate of *Leptolegnia sp.* on various pH media ; pH4 (●), pH5 (▲), pH6 (■), pH7 (⬢) and pH8 (♦) determined by colonial diameters. Results represent the average of triplicate cultures.

(B). Growth of 36A-LK isolate on various pH media determined on day 1 (●), day 2 (▲), day 3 (■), and day 4 (⬢) of cultures by colonial diameters.

Table 36 Carbon assimilation test of mosquito larvae killing fungi

Fungus	Carbon assimilation				
	Glucose	Maltose	D-galactose	Lactose	Saccharose
12A-6 *Trichoderma viride*	+	+	+	+	+
10A-15W *Beauveria sp.*	+	+	+	+	+
29B-5W *Aspergillus niveus*	+	+	+	+	+
35C-1 *Aphanomyces sp.*	+	-	-	-	-
36A-LK *Leptolegnia sp.*	+	+	-	-	-
Candida albicans	+	+	+	-	+
Candida krusei	+	-	-	-	-
Aspergillus flavus	+	+	+	+	+

aquatic fungus, 35C-1 isolate (*Aphanomyces sp.*) assimilated only glucose and 36A-LK isolate (*Leptolegnia sp.*) assimilated glucose and maltose.

2.3.2 Nitrogen assimilation test

Nitrogen sources were urea, asparagine, ammonium sulphate, sodium nitrate and peptone (Table 37). *Rhodotorula glutinis* which could assimilate all nitrogen sources was used as the positive control while *Torulopsis glabrata* which could assimilate only peptone was used as the negative control. All solates of mosquito larvae killing fungi found in this investigation could assimilate peptone. Three isolates, 12A-6 isolate (*Trichoderma viride*), 10A-15W isolate (*Beauveria sp.*) and 29B-5W isolate (*Aspergillus niveus*) could assimilate all nitrogen sources whereas two isolates of aquatic fungi, 35C-1 (*Aphanomyces sp.*) and 36A-LK (*Leptolegnia sp.*) could assimilate only peptone.

Table 37 Nitrogen assimilation test of mosquito larvae killing fungi

Fungus	Nitrogen assimilation				
	Urea	Asparagine	Ammonium sulphate	Sodium nitrate	Peptone
12A-6 *Trichoderma viride*	+	+	+	+	+
10A-15W *Beauveria sp.*	+	+	+	+	+
29B-5W *Aspergillus niveus*	+	+	+	+	+
35C-1 *Aphanomyces sp.*	-	-	-	-	+
36A-LK *Leptolegnia sp.*	-	-	-	-	+
Rhodotorula glutinis	+	+	+	+	+
Torulopsis glabrata	-	-	-	-	+

DISCUSSION

Mosquito larvae samples collected from 100 aquatic habitats in the northern Thailand provinces were found to be infected with fungus only 1.1%. The low percentage of infection, may be due to the setting down of fungal infected cadavers to the bottom of the water. Thus, almost all uninfected mosquito larvae that remained close to the surface of water for feeding were sampled. The epizootic of fungal infection in mosquito larvae was rarely found in natural aquatic habitat. Only 1-3 mosquito larvae infected with fungus were always found in each sampling. In this survey, the percent fungal infection in *Culex* mosquito larvae was high (77.5%) whereas it was 8.2% for *Aedes sp.*. This was due to the prevalence of *Culex* and *Anopheles* in aquatic habitats. The probability to find mosquito larvae killing fungi from natural aquatic habitats was higher than that from artificial container in which *Aedes sp.* perferred to live.

Almost all the isolated fungi were saprophytes including soil fungi and aquatic fungi. The first fungus which showed the mosquito larvae killing ability was *Trichoderma viride.* This fungus was previously reported as fungal pathogen of mosquitoes in 1949 by Steinhaus (cited in Roberts, 1977). In this study, *Trichoderma viride* killed 4 species of mosquito larvae. Nevertheless, anopheline larvae were less susceptible in compare

with *Culex quinquefasciatus*. The differences in susceptibility may be due to the sinking down of the spores to the bottom of the assay petri dish where they may be more accessible to the bottom-feeding *Culex* and *Aedes* than to the surface-feeding *Anopheles*. Thus, *Culex* and *Aedes* larvae tend to ingest more fungal spores than anopheline larvae. Formulation development will be a most important expectation in the future. The fungus needs to be produced in a stable form that can be efficiently directed at the target species. The ability of spores to sink in water may be advantageous for use against some bottom feeders such as *Aedes* and *Culex* larvae but it may be necessary to formulate them so that they or some portion remain close to the surface for control of *Anopheles*. The sticky substance coating the spore which permits adhesion to the cuticle may be utilized for this purpose.

At higher dose many more larvae died without fungal colonization of the bodies. It appeared that the increase in dosage of conidia had a detrimental effect on mosquito viability although there was no fungal invasion. Goettel (1987) also found a marked difference between percent mycosis and percent mortality in mosquito larvae exposed to *Tolypocladium cylindrosporum* conidia. The presence of ungerminated conidia in the guts of mosquitoes may be detrimental. In *Culicinomyces clavisporus*, death occurs within hours of ingestion of large quantities of conidia (Panter and Russell, 1984). It was speculated that death was the result of

release of toxins by germinating conidia. In this study, initial observation of rapid death was made during an experiment to determine whether the dead spores were toxic to larvae. Autoclaved spores at concentration of 10^5 - 10^7 conidia/ml did not kill mosquito larvae much more than the control, while live spores at the highest concentration caused substantial kill within 5 days. This phenomenon of rapid death was under further investigation, with a toxin being suspected as the likely cause. Thus, filtrates of one week broth cultures of 12A-6 isolate of *Trichoderma viride* were tested with *Aedes aegypti* larvae. Only thirty five percent of larvae were killed, suggesting that the mortality observed in the bioassays was due to the presence of spores and might be not the result of a toxic compound in the culture medium. It was likely that mosquito larvae were killed when the mycelium penetrated the cuticle and invaded the mosquito larvae tissues.

Some isolates of mosquito larvae killing fungi, 10A-15W isolate of *Beauveria sp.* and 29B-5W isolate of *Aspergillus niveus* were shown to produce compounds toxic to mosquito larvae. When filtrates of one week broth cultures of 10A-15W and 29B-5W were tested *Ae.aegypti* larvae, they gave 97.5 and 77.5% mortality, respectively. In summary, symptoms of mycosis in insect with fungi limited colonization prior to death and paralysis of hosts were consistent with toxicosis as the cause of death (Samuels *et al.*, 1988). Various kinds of fungi were isolated as causes of insect

disease. However, some of them were known to produce toxins which may play an important role in mycosis. Ohtomo and co-workers(1975) suggested that aflatoxin B1 produced by *Aspergillus flavus* might cause the *Aspergillus* disease of silk worm. There were many authors reported the incidence of *Aspergillus sp.* infection in mosquitoes (cited in Roberts, 1977) such as Christophers (1952), Speer (1927), Galli-Velerio and Rochaz de Jough (1905) found the incidence of this fungus infection in *Anopheles* and *Culex* larvae, in 1959, Laird found *Aspergillus* infection in *Culex* larvae and in 1965, Hati and Ghosh found that *Aspergillus parasiticus* infected in *Anopheles* in India. However, none of them studied how toxin might play a role in mosquitoes'mortality. Conidia of *Culicinomyces clavisporus* a virulent fungal pathogen of mosquito larvae (Couch *et al.*, 1974), are normally ingested during feeding. They germinate in the foregut or hindgut, and cause death when invading hyphae fill the haemocoel (Sweeney, 1975). However, with high doses of fungus (10^4-10^6 conidia/ml), death can occur within 18 hrs to 2 days without extensive growth of the fungus in the larvae, which leads to a suggestion that a toxin may be involved (Couch *et al.*, 1974; Panter and Russell, 1984). Moreover, *Metarhizium anisophiae* was reported to kill insect larvae by production of toxin called destruxin A and B which were present in submerged cultures (Roberts, 1969). This compounds induced paralysis immediately after infection in larvae of the greater wax

moth, *Galleria mellonella* and in tobacco hornworm, *Manduca sexta* (Samuels *et al*,. 1988). Certain strains of the fungi *Beauveria bassiana* can produce *beavericin* which is a cyclodepsipiptide with insecticidal properties (Grove and Pople, 1980; Peeters *et al.*, 1983). At present, beavericin is also synthesized (Peters *et al.*, 1983; 1988) by a soluble enzyme system from D-~~L~~-hydroxyisovaleric acid and phenylalanine under consumption of ATP and S-adenosyl-L-methionine. The production of a toxic substance bassianolide, was also detected in the fungal bodies of *Beauveria bassiana* and *Verticillium lecanii* (Murakoshi *et al.*, 1978; Suzuki *et al.*, 1977) and it caused atonic symptoms in the silkworm larvae. Bassianolide could inhibit smooth muscle contraction (Nakajyo *et al.*, 1982). Isolates of 12A-6 *Trichoderma viride* and *Aphanomyces sp.* were less virulent and took longer time to kill mosquito larvae than 10A-15W of *Beauveria sp.* and 29B-5W of *Aspergillus niveus.* Thus, high toxin production may not be essential for the pathogenesis but may speed up the disease process (Samuels *et al., 1988).* Moreover, some mosquito larvae showed developing infections of fungus within the body cavity whereas the others appeared to be free of the fungus. This suggests that some of larvae were able to molt before the fungus was established within them and that they were able to cast off the infection with the discarded cuticle. This phenomenon was observed in laboratory experiments with *Culicinomyces clavisporus* (Sweeney *et al., 1983).*

Beauveria sp. had ability of killing mosquito larvae as other authors reported (Pinnock *et al.*, 1973; Clark *et al.*, 1968). *Beauveris tenella* was found to be a naturally occuring pathogen in field population of *Aedes sierrensis* larvae (Pinnock *et al.*, 1973). The bioassay showed that *B. tenella* was also pathogenic for larvae of *Aedes aegypti*, *Ae.dorsalis*, *Ae.hexodontus*, *Culex pipiens*, *Cx.tarsalis*, and *Culiseta incidens*. Meanwhile, 10A-15W isolate of *Beauveria sp.* was found to kill *Anopheles dirus* and *Anopheles minimus*. The amount of this isolate required to achieve high mortality (more than 50%) for *An.dirus* was considerably less than that required for either the *Culex* or the *Aedes* mosquito. Because *An. dirus* is a surface feeder while both *Cx. quinquefasciatus* and *Ae.aegypti* are depth feeders and are usually found well beneath the water-air interface. The difference in susceptibility between the species might be attributable to the fact that 10A-15W spores are hydrophobic by nature and present at very high dosage levels at the water's surface where *An.dirus* are found. However, the dose-response curves for *Cx.quinquefasciatus* and *Ae.aegypti* are the result of either more chance contact with the floating spores as their density increases or the relative numbers of submerged spores increasing which raised the number of spores consumed by the larvae beneath the surface (Ramoska *et al.*, 1981). The different result was achieved by Clark and co-workers (1968). They reported that the larvae of the species of *Aedes*

were not susceptible to floating conidia of the strains of *Beauveria bassiana* assayed, possibly due to the longer time necessary for infection. They also found that timing of the conidiospore application was critical; those larvae avoiding contamination during the first day after molting avoided fatal infections during that larvae stage.

In the case of *An.minimus* which less susceptible to 10A-15W isolate of *Beauveria sp.*, it could be suggested that the difference in susceptibility of mosquito larvae to this fungus might be due, at least in part, to differences in detoxifying ability (Samuels *et at.*, 1988).

In our study, we found the infection of mosquito larvae by *Aphanomyces sp.* There was an occurrence of *Aphanomyces* (Saprolegniales: Fungi) infections in laboratory colonies of larval *Anopheles* (Seymour *et al.*, 1984). Seymour and Briggs (1985) reported that investigations in Maxico and Nigeria could detect the mycoses in larval maintenance units for *Anopheles albimanus* Wiedemann at the Centro de Investigation de Paludismo, Tapuchula, Chiapas, Mexico, in 1983, and in larval maintenance facilities for *An.gambiae* Giles at the National Arbovirus and Vectors Research Unit, Enugu, Anambra State, Nigeria, in 1982. Like most watermolds, *Aphanomyces sp.* are common in both terrestrial and fresh water habitats. Introduction of *Aphanomyces* in the Mexican' colonies was found to be *via* tap water used in culturing the larvae. In

Nigeria, untreated rainwater collected as runoff from the laboratory and stored in open containers contained dead insects infested with *Aphanomyces*, which was then introduced into the rearing pans. A second source of contamination in the Nigerian facility was through soil-infested roots on plants used as infusion for *An.gambiae*. However, no other reports trying to use *Aphanomyces* for killing mosquito larvae. This may be due to the short period of pathogenicity of this fungus against mosquito larvae. We found that it was difficult to prolong its infectivity. After 2-3 passages in mosquito larvae, its infectivity was lost. The 35C- 1 isolate of *Aphanomyces* yielded a small percent mortality when the culture was used as inoculum. This might be due to the failure of zoosporogenesis in this condition. Zoospores were the infective form of aquatic fungi in mosquito larvae infection (Lord and Fukuda, 1988; Umphlett and Huang, 1972). Unfortunately, specific identification of the fungus was not possible since the sexual reproductive structures essential for species determinations within the genus were not formed by any of the isolates either naturally or by culture manipulation.

Among the difficulties associated with culture maintenance of larval mosquitoes was the unpredictable apperance of mycotic infections caused by pathogenic zoosporic fungus, 36A-LK isolate of *Leptolegnia sp.* The resulting epizootics, although commonly lethal and disruptive, are generally of brief duration in occurrence as reported for the other aquatic fungi (Seymour and Briggs, 1985).

An aquatic fungus *Leptolegnia sp.* has been previously reported to be pathogenic to mosquito larvae and to show potential as a biocontrol agent for medically significant insects (McInnis and Zattau, 1982; Seymour, 1984). Mosquito larvae of *Culex pipiens*, *Cx. salinarius*, *Culiseta inornata*, *Aedes taeniorhynchus*, and *Ae. triseriatus* were susceptible, and the mortality exceeded 80% in all species while we found that *Ae.aegypti* could also be infected. *Leptolegnia* shows a high degree of specificity for mosquito larvae among arthropods but little specificity among the target species (McInnis *et al.*, 1985). Lord and Fukuda (1988) elucidated its mode of infection at the ultrastructural level that the pathogen revealed 2 differences portion of entry by penetration *via* the gut and penetration *via* the integument. The latter often involved aggregations of zoospores, appressoria-like swellings of the invasive hyphae, and lateral growth of hyphae between the epicuticle and endocuticle. These features were not detected in the case of gut invasion, but hyphal septa at the point of entry were apparently peculiar to this route. There was no evident of tissue specificity, and death presumably resulted from generalized destruction of tissues. Although these data appear promising for further development of *Leptolegnia* for control purposes and microscopic examination verified that nearly all of the mortality was due to fungal infection. However, a decreasing effectiveness of the pathogen was noted as culture of *Leptolegnia sp.* on artificial

media was used as inoculum. The cause for this failure is not exactly elucidated but probably comes from the decline in pathogenicity of our isolate from prolonged maintenance on artificial media as suggest by McInnis and co-workers (1985). However, it was previously reported that although this fungus was relatively easy to culture and manipulate *in vitro*, it was not considered sufficiently active to warrent further study as a mosquito larvicide. First, because it lost its larvicidal activity upon prolonged culture (cited in Nnakumusana, 1986). Second, the reduction of infection rates may be due to failure to complete zoosporogenesis on artificial media, resulted in no or small amount of zoospores which were an infective stage as described previously. Moreover, we found that the percent mortality was higher when infected cadavers were used as inoculum and zoospores could be found only on the mosquito cadavers. Nevertheless, carcasses were not useful for inoculum if they died for more than 24 hrs since the zoospores discharge proceeded very rapidly after that time. Forty eight hours after the onset of sporulation, the spore production capacity of the fungus in the carcass was exhausted (Umphlett and Huang, 1972). We found the problem about how to prolong the infectivity of *Leptolegnia sp.* which related to zoospores production. The variability of the data for zoospores produced *in vivo* probably reflects the condition of the fungus in its parasitic state. When infected *Aedes aegypti* were the zoospore

source, some of the mosquito cadavers failed to produce zoospores. This was probably due to bacterial invasion through the wounds caused by heavy fungal infection as suggested by Lord and co-workers (1988) or because the tested larvae began eating the cadavers used as inoculum before the fungal sporangia had an opportunity to develop the infective zoospores (McCray *et al.*, 1973). Thus, it would lose its infectivity and could not prolong its infectivity.

The zoospore production and infectivity were greatly improved by supplying aquatic fungal culture with a sterols rich medium or hemp seed extract medium (Hemp seeds have a high content of sterols which are a major requirement for zoospore production) (Domnas *et al.*, 1982; 1977). In 1981, Nnakumusana and Seymour reported that zoospore production of *Leptolegnia sp.* from either cornmeal or potato dextrose was less than that from a single half heep seep. They also suggested to culture this fungus on medium in which mosquito extracts was added because mosquito extracts could induced varying degree of oospore germination within 24-72 hrs and there was a paper reported the using of oospores of aquatic fungus as a potential agent (Kerwin and Washino, 1986). This increases the chance of *Leptolegnia* to become a candidate biological control agent against mosquito vectors. Moreover, some isolates could infect mosquito larvae in water with greater than 5 ppt (part per thousand) of NaCl (Lord *et al.*, 1988). *Leptolegnia sp.* infected mosquitoes in water with NaCl concentration well above the tolerance

limits of *Lagenidium giganteum*. However, one disadvantage noted was that *Leptolegnia sp.* killed 100 percent *Toxorhynchites brevipalpis* (Nnakumusana, 1986) which were useful for their predatory habits on mosquito larvae of other genera. It would be an added advantage if this fungus could be integrated with the predator to keep the mosquito larval population down.

Other mosquito larvae killing fungi which were not selected for studies were also previously found to have mosquito larvae killing ability. Bacinskij (1926) reported the infection of *Penicillium glaucum* in *Culex* mosquito larvae (Cited in Roberts, 1977). It was reported that the metabolites of *Penicillium sp.* caused weight reduction and mortality of the lepidopterous larvae *Spodoptera littoralis* (Egyptian cotton leafworm) and the fruit fly *Drosophila melanogaster* (Paterson *et al.*, 1987). In this study, we found that *Paecilomyces sp.* also killed mosquito larvae. Bernardini and co-workers (1975) previously reported that they could isolate beauvericin from *Paecilomyces fumoso-roseus*. This toxin might contribute the killing ability of *Paecilomyces sp.* as similar to what it does in *Beauveria sp.* Microscopic observations of test larvae exposed to the fungal inoculum revealed that mycelial growth occured in the head, abdomen, anal segment or gills. It is likely that the spores are normally ingested and probably enter the tissues of the host larva through the digestive tract or by attachment and penetration of the exoskeleton as described by other

authors (McCray *et al.*, 1973, Soares, 1982). Sweeney (1975) found that most spores pass through the gut in an apparently unchanged condition and some spores were ingested without any adhering to the alimentary tract. The cause of death in mosquito larvae might be the complete packing of haemocoel with hyphae as occurs with *Lagenidium giganteum.* The physiological starvation caused by a depletion of host nutrient reserves was then established (cited in Soares, 1982). The disease development process can be devided into 10 steps. A brief discussion of these steps as understood in 1979 was presented by Roberts and Humber in 1981 and recounted by Roberts and Aist (1984) as follows:

1. Attachment of the conidium to the insect cuticle. The conidia of entomopathogenic fungi are apparently adapted for attachment to insects. The physical and chemical characteristics of conidial and cuticular surfaces responsible for attachment are unknown. The importance of this step for infection was demonstrated with a mutant of *Metarhizium anisopliae* which was hypovirulent for mosquito larvae. In comparison to the virulent wild type, very few conidia of the mutant attached to the perispiracular valves of the *Culex pipiens* larvae.

2. Germination of the conidium on the insect cuticle. In general, high relative humidity is needed for germination. Both germination stimulators and inhibitors have been reported to be on the cuticle. Bacteria on the cuticle can inhibit the germination of

M.anisopliae.

3. Penetration of the cuticle. The germ tube may penetrate directly into the cuticle. An appressorium which attached firmly to the cuticle may be formed and a narrow infection peg was sent into the cuticle. Cuticular invasion involves both enzymatic and physical activities.

The enzymes elaborated by germinating conidia have not been identified, but it is known that colonies of most entomopathogenic conidial fungi produce proteases, lipases, and chitinases in liquid and agar media. With *Beauveria bassiana,* these enzymatic activities did not dissolve excised insect cuticle when applied individually, but the cuticle was dissolved by the mixture. Insect which molt before the hemocoel is invaded may discard the fungus totally in the molting process.

4. Growth of the fungus in the hemocoel. The fungus usually grows in the hemocoel as yeastlike hyphal bodies, essentially blastospores, which multiply by budding. In some instances, hyphae rather than hyphal bodies occur in the hemocoel.

5. The production of toxins. Many entomopathogenic fungi overcome their hosts before extensive invasion of organs takes place, and toxins are presumed to be responsible for host mortality. Although compounds toxic to insects have been reported from culture filtrates and mycelia of several entomopathogenic fungi and from fungi not known to affect insects naturally. However, the

depsipeptides destruxin B and desmethyldestruxin B were detected in *Metarhizium anisopliae* infected silkworm larvae at levels known to be lethal to this species. All presently known toxins were isolated and identified from mycelia or culture filtrates of fungi grown *in vitro*. The majority are small molecules, primarily depsipeptides. Enzyme, particularly proteases, are present in culture filtrates. When concentrated, these are detrimental to certain insects on intrahemocoelic infection, but their role in disease development is unknown. The extensive fungal development prior to death of some fungus-insect combinations indicates that toxins are not produced.

6. Death of the host. This may be preceded by behavior changes such as tremors, loss of coordination, or climbing to an elevated position

7. Growth in the mycelial phase with invasion of virtually all organs of the host. Because of the replacement of internal organ with mycelia, the dead insect may appear almost normal for a while. Small, melanized spots at the sites of infection may be apparent. In some cases a reddish cast (*Beauveria bassiana*) or blackening (*Entomophthora*) of the host is detected. In addition to hyphal filaments, chlamydospores may be produced. These fungus-filled insects ("mummies") can serve as reservoirs of the fungus through periods of adverse conditions, such as dry or cold weather.

8. Penetration of hyphae from the interior through the cuticle to the exterior of the insect. If the "mummy" is held under

conditions of moderate or low relative humidity, the fungus will remain within the cadaver. The fungus will grow through the cuticle when the cadaver is placed in a humid environment. The intersegmental membranes which afford less resistance to penetration, is where most reemergence of fungi, particularly the Entomophthorales occurs.

9. Production of infective units (usually conidia) on the exterior of the host.

10. The final step is the dispersing of infective units to locations where they are likely to encounter susceptible insects for the initiation of new cases of disease. The spores of the Entomophthorales are forcibly discharged, while the conidia of other entomopathogenic fungi are passively dispersed.

Although there are many fungi, having mosquito larvae killing ability, the reduction of infectivity of these fungi was the main problem. it was suggested that pathogenicity of these fungi might be reduced during repeated subculturing on agar. While the entomogenous fungi have a saprophytic phase in dead insects and can be cultivated on artificial media, the parasitic phase in their life cycle without loss of virulence cannot be omitted (cited in Schaerffenberg, 1964). We do not know at this time how long-that is through how many generations-an entomogenous fungus can be cultivated in artificial substrates without losing its virulence. For *Beauveria bassiana*, the virulence was maintained on

approximately the same level through 16th nutrient medium generation. From there on the death rate gradually decreased until in the 30th nutrient medium generation only 50 percent of the experimental insects were killed (Schaerffenberg, 1964). Therefore, in order to preserve its full virulence, *B.bassiana* must again be given an opportunity to complete its growth cycle by passing it through insect by the 15th, but at the lastest by the 16th, nutrient-medium generation. Such problems underline the importance of maintaining stock cultures of pathogens which are biological identical, or similar, to the parent stock, and which can be stored for extended periods without deterioration. It is advisable to store isolates of fungus by storing numerous agar cultures on spore suspensions in, for example, deep-freeze or liquid nitrogen, after as few generations as possible beyond isolation from the original insect host. Such meterial can then be activated when required to produce cultures for experimental purposes (Hall, 1980).

The mosquito larvae killing tested by bioassay had many factors affecting the variability of assay results (Goettel, 1987), there is no standard assay which seem to be more suitable and give less variable results at present. The rate of development of the host and the time necessary for the fungal pathogen to infect and become established are affected by the temperature of the assay system (cited in Fargues and Remaudiere, 1977). Differences between batches of mosquito larvae and fungal pathogen as well as

inaccuracies in the estimation of the dose are usually the principle sources of variation in bioassays. We found that isolated fungi had broad range specificity in killing when screening test was performed. In some cases, the 50% lethal concentration (LC 50) could not be calculated in further bioassays eventhough the percent mortality in the screening test was more than 50. We suggest that the inoculum in screening test might be more than 10^7 spores/ml which was the highest concentration used in bioassays. The state of mosquito larvae used in bioassay also had an influence on susceptibility to the fungal pathogen. Umphlett and Huang (1972) found that a decreasing effectiveness of the fungal pathogen was noted as larval age increased. Young larvae were more susceptible than older larvae (Pinnock *et al.*, 1973). Goettel (1987) suggested the use of very young larvae in order to decrease sources of variability in bioassay. The data were highly heterogeneity when older instars were used and heterogeneity was reduced when younger instar larvae were used. The reduction of heterogeneity was probably a result of the more uniform physiological state of the younger larvae. Cooper and Sweeney (1982) suggested that *Aedes aegypti* might be the most suitable test mosquito larvae as the less variable results tend to render a greater precision of the LD 50 estimated. They found the smaller with in assay heterogenicity recorded in those experiments using *Ae.aegypti*. This might be due to the normal container breeding habit of this species. These larvae response more uniformly in the assay

containers than those of the other species, with a consequent reduction in assay variability.

Goettel (1987) suggested that mosquito larvae were exposed to the inoculum for a limited time in order to standardize the dose and decrease sources of variability in bioassay. The larvae are contineously exposed to the inoculum because of the closed system assay. Hence, the effective dose varies to the time of exposure as mosquitoes reingest the conidia again and again. Futhermore, ingestion of conidia is presumably not contributing to the nutrition of the mosquito. It is probable that the larvae at each higher conidial concentration are put under increasing stress as they ingest more and more non-nutritive matter (i.e., conidia) and there by become more susceptible to mycosis. Longer exposure times will also tend to increase variability between replicates and doses because of the random contamination by microorganisms from the air and the larval mortalities that subsequently affected the amount of food in each container. The bioassay is further complicated since each assay petri dish rapidly becomes a habitat of its own with a different microbial funna. It most likely has an effect on the viability of the conidia (Goettel, 1987).

However, for a potential biological control agent, the successful demonstration of its pathogenicity towards mosquito larvae is not alone sufficient unless the agent is also amenable to mass production. Hence, the optimal condition for growth was

studied. It was found that there was no proportional increase in spores with increasing mycelial yield such as 12A-6 isolate of *Trichoderma viride*. It was found no difference in fungal growth when cultured on CMDP and SDA by colonial diameter measuring. The dry weight yeilded significantly highest on SDA ($p<0.05$) whereas spore production on SDA was least. Increasing concentrations of glucose in SDA improved mycelial yield but repressed spore production. Schaerffenberg (1964) reported that higher carbon content appears to promote the mycelium growth while an inversely higher nitrogen content appears to encourage the formation of conidia. It was likely that ascospore production might also be depended on nutrient in each media. For 29B-5W isolate of *Aspergillus niveus*, ascospores were detected instead of conidia because of this fungus produced less conidia comparing with ascospore. Moreover, its spore was very small and difficult to enumerate. The cultures of this fungus can be transferred by means of conidia, ascospores or hyphal tips. If repeated transfers are made by conidia only, the ability to form ascospores declines, but can be restored by making subcultures from ascospores (Mather and Jinks, 1958).

Among 3 methods using for fungal growth determination, spore enumeration was the most variable (8.8% variation). The suitable methods for mosquito larvae killing fungal growth determination should be chosen according to its infective form. For

example, conidia was the infective form of *Beauveria sp.* as reviewed by Roberts and Aist (1984). Thus, the spore enumeration should be used to detect the optimal condition for growth of this fungus. The mass production of infective stage of mosquito larvae killing fungi was necessary for further field application.

The optimal temperature for growth and spore production of 12A-6 isolate of *Trichoderma viride* was 20-25°C. It was similar to the previous report reviewed by Domsch and co-worker (1986). This fungus was unable to grow at 37°C. Low temperature (10°C) retarded the growth and spore production. The temperature of mosquitoes breeding sites ranges from 18°C occasionally to 37°C. The results of this study indicate that temperature would be a limiting factor achieving *Trichoderma viride* growth in some but certainly not all habitats. This fungus grew in wide range of pH (1.5 - 9.0) and the optimal being in the range pH 4.5 - 5.5 (Domsch *et al.*, 1986). While in our study, it grew in all pH tested but slowly at pH 8.

The 10A - 15W isolate of *Beauveria sp.* easily grew in culture. It grew in all testing media and in wide pH range. Moreover, it assimilated all carbon and nitrogen source in this test. However, this isolate cannot grow within a wide temperature range. It grew well at 20-25°C and was unable to grow and sporulate at 37°C Walstad and co-workers (1970) reported that *Beauveria bassiana* were unable to sporulate at temperatures below 10°C or above 35°C

and the most rapid sporulation occured at 25°C while in this study, the maximum spore production was at 20° C. Nevertheless, the infection of *Beauveria* are not extensively temperature dependent. They can normally be developed within a wide temperature range which extends from 0 to 40° C (cited in Schaerffenberg, 1964). Although *Beauveria sp.* appeares to be more potentially mosquito larvicidal, the pathogenicity of this fungus demonstrated against some of animals. It caused pathogenicity in the rabbit cornea (Ishibashi *et al*,. 1987), eventhough keratitis in human (Sachs *et al.*, 1985). This could also present a risk, the future of this agent as a potential mosquito larvicide remains uncertain. This non-specificity may be a problem that hinders the use of this and other deuteromycetous fungi in aquatic ecosystems.

The 29B - 5W isolate of *Aspergillus niveus* grew and sporulated well at high temperature (25 - 37°C) but low temperature suppressed its growth. Although the optimal temperature for growth reported by Borut in 1960 (cited in Domsch *et al.*, 1986) was determined at both 36°C and 41-42°C, this fungus appears to be more tolerant in high temperatures. For the aquatic fungi, 35C-1 isolate of *Aphanomyces sp.* grew at all temperatures and pH tested but at 10°C and at pH4, the growth rate was slow. Yang and Schoulties in 1972 (Cited in Domsch *et al.*, 1986) reported that retarded development occured below 15° C while the optimal temperature for vegetative growth was in the range 25-30° C. This isolate of *Aphanomyces sp.*

can be usually exposed to more than 30°C, the typical summer temperature of northern Thailand. This could in part account for its growing ability at 37 °C in the laboratory. Furthermore, Yang and Schoulties also found that good growth was obtained in the pH range 5.2 - 7.7 and optimal growth at pH 5.4 - 6.5. The growth did not occur below pH4 or above pH8. The optimal temperature for culture growth of 36A-LK isolate of *Leptolegnia sp.* was 20-25°C while it was 25°C as reported by Nnakumusana (1986). The lower temperature (25 °C) suppressed growth of *Leptolegnia* and at 30°C the growth rate was very slow and there was no growth at 35° C. In this study, *Leptolegnia sp.* grew at 10°C and 37°C but the growth rate was slow. The advantage of this isolate was the wide temperature range of its growing ability. However, this fungus was most virulent at 25°C, but had no infectivity at 35°C (Nnakumusana and Seymour, 1981). The optimal pH for growth was 6 - 8, similar to the previous report which also found that *Leptolegnia* was the most virulent at this pH range but ineffective at pH below 5 and pH above 10 (Nnakumusana, 1986).

Almost of the mosquito larvae killing fungi in this study were saprophytes which were easily grown in artificial media (Gardner and Pillai, 1986) and assimilated all carbon and nitrogen source tested. The aquatic fungi, *Aphanomyces sp.* and *Leptolegnia sp.* assimilated only peptone as nitrogen source. They could not assimilate inorganic nitrogen. For this reason, nitrogen source in

basal medium for carbon assimilation test was changed from ammonium sulphate to peptone. The inability of fungi to assimilate a substrate is the result of either the substrate's not entering the cell or the fungus' lacking the appropriate enzyme(s) needed to convert the substrate into an intermediate compound of a central metabolic pathway (McGinnis, 1980).

This studies provide basic information needed for further studies of the potential of these fungi as an agent for biological control of mosquito larvae. There are several questions that require answers before effective use of the fungus for biological control is possible. Factors such as its infectivity, portal of entry, tissues or organs primarily attacked, and mode of infection are basic to an understanding of the parasite-host relationship. In addition to basic biological studies, methods for production and storage of the infective material must be developed, the effects of varying environmental conditions and their safty to non target organisms must be determined, and infectivity and virulence for appropriate mosquito species must be assessed before the pathogen is selected as a candidate for field experiments. Alternatively, further research could be undertaken either to enhance the virulence of the isolate of mosquito larvae killing fungi currently avialable or to increase the search for more virulent isolates.

Summary

Mosquito larvae samples were collected from 100 aquati habitats in the northern Thailand provinces. After microscopic examination, forty nine mosquito larvae were found to be infected by fungi. Thirty two isolates of fungi included 4 isolates of *Aspergillus niger*, 4 of *Penicillium sp.*, 3 of *Fusarium sp.*, 3 of *Trichoderma sp.*, 2 of *Cladosporium sp.*, 2 of *Paecilomyces sp.* and 1 each of *Curvularia sp.*, *Aspergillus niveus*, *Beauveria sp.*, *Aphanomyces sp.* and *Leptolegnia sp.* were isolated from these infected cadavers. Nine isolates of fungi were unidentified. The screening tests of mosquito larvae killing ability of these isolated fungi against laboratory colonized mosquito larvae showed that only 10 isolates exhibited more than 50% mortality at a given concentration. However, three isolates from these were selected according to their high virulence (low LC_{50}) and obvious showing sign of infection in mosquito larvae.

The isolate No.12A-6, *Trichoderma viride*, isolated from *Culex sp.* in Chiang Mai province killed larvae of *Cx. quinquefasciatus*, *Ae. aegypti*, *An. dirus*, and *An. minimus* with LC_{50} values at concentration of 7.16×10^5, 1.29×10^6, 1.40×10^6, and 1.71×10^6 spores/ml, respectively. This fungus grew fast on CMDP and PDA. The dry weight was highest on SDA while the spore production was highest on CMPD. The optimal temperature for growth

This fungus could assimilate all of the carbon sources tested, including glucose, maltose, D-galactose, lactose, and saccharose. It also assimilated all of the nitrogen source tested, including peptone, urea, asparagine, sodium nitrate, and ammonium sulphate.

Second, 10 A - 15 W isolate of *Beauveria sp.* isolated from *Culex sp.* in Chiang Mai province killed larvae of *Cx. quinquefasciatus, Ae. aegypti, An. dirus,* and *An. minimus* at LC 50 2.43 x 10^6, 2.92 x 10^6, 1.18 x 10^6, and 3.96 x 10^6 spores/ml, respectively. The culture filtrate of this fungus could also kill the mosquito larvae. Thus, a toxin might play an important role in killing ability. This isolate was slow-growing fungus. On SDA, the fungal growth rate was slowest while the dry weight was highest. The spore production of 14 days old cultures on PYG and CMDP were higher than those on other media. The optimal temperature for growth was between 20-25°C and for spore production was 20°C. The optimal pH for growth was ranging from 6 to 8 while the spore production of a week culture was high at pH 7 and pH 8. Furthermore, this fungus could assimilate all of the carbon and the nitrogen sources tested.

Third, 29B - 5W isolate of *Aspergillus niveus* isolated from *Culex sp.* in Nan province killed larvae of those 4 species of mosquito larvae at LC 50 1.79 x 10^4, 1.59 x 10^5, 6.73 x 10^4 and 3.86 x 10^5 spores/ml, respectively. The culture filtrate of this fungus could also kill mosquito larvae. Thus, a toxin might

contribute to this killing ability. This fungus was able to grow on SDA, CMDP, and PDA. The growth rate on PYG was found slowest. When cultured on SDA, the dry weight was highest whereas the ascospore production was high on CMDP and PDA. The optimal temperature for growth and the ascospore production of this fungus was between 25-37 °C. It grew quite well in wide range of pH (5-8) and the ascospore production was very high at pH 5-6. This fungus also assimilated all of the carbon and the nitrogen source tested.

There were two interesting isolate of fungi which, one was 35C-1 isolate of *Aphanomyces sp.* isolated from *Culex sp.* in Sukhothai province. The other was 36A-LK isolate of *Leptolegnia sp.* isolated from *Aedes aegypti* in Chiang Mai province. Both isolates were aquatic fungi which infected mosquito larvae despite the percent mortality caused by these isolates was less than 50. The percent mortality would be raised if infected cadavers were used as inoculum in bioassay instead of the fungal culture.

The 35C-1 isolate of *Aphanomyces sp.* was fast-growing and grew in all testing media. However, the dry weight was highest on SDA. This isolate grew well at 20-37°C but the growth rate at 25°C was very fast. Moreover, it also grew well at all pH tested except at pH 4. It assimilated peptone as nitrogen source. For carbon source, this fungus assimilated only glucose.

The 36A-LK isolate of *Leptolegnia sp.* easily grew on all testing media but yielded highest dry weight on SDA. This fungus

grew at all temperatures tested and rapidly at 20-25°C. The optimal pH for growth ranged from pH 6 to pH 8. This fungus assimilated peptone as nitrogen source, glucose and maltose as carbon source.

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VIII. APPENDIX

1. Cornmeal dextrose peptone agar (CMDP)

Formulation :

Corn meal agar	17 gm
Dextrose	10 gm
Peptone	2 gm
Distilled water	1 liter

Preparation :

a. Mix reagents.

b. Bring to a boil.

c. Autoclave for 15 minutes at 15 psi.

d. Dispense into sterile Petri dishes.

e. Allow to harden.

2. Sabouraud's dextrose agar (SDA)

Formulation :

Peptone	10 gm
Dextrose	20 gm
Agar	15 gm
Distilled water	1 liter

Preparation :

a. Dissolve the ingredients by boiling.

b. Autoclave for 10 minutes at 15 psi.

c. Dispense into sterile Petri dishes.

d. Allow to harden.

3. Sabouraud's dextrose broth (SDB)

Formulation :

Peptone	10 gm
Dextrose	20 gm
Distilled water	1 liter

Preparation :

a. Dissolve ingredients.

b. Dispense 100 ml of this medium into 250 ml erlenmeyer flasks.

c. Autoclave for 10 minutes at 15 psi.

4. Peptone yeast-extract glucose (PYG)

Formulation :

Peptone	1.2 gm
Yeast extract	1.2 gm
Glucose	3 gm
Agar	15 gm
Distilled water	1 liter

Preparation :

a. Dissolve the ingredients by boiling.

b. Autoclave for 10 minutes at 15 psi

c. Dispense into sterile Petri dishes.

d. Allow to harden.

5. Lactophenol cotton blue (LPCB) (Larone, 1987)

LPCB is used as both a mounting fluid and a stain. Lactic acid acts as a clearing agent and aids in preserving the fungal structures ; phenol acts as a killing agent ; glycerol prevents drying ; and cotton blue gives color to the structures.

Formulation :

Lactic acid	20 ml
Phenol crystals	20 g
(or phenol, concentrated	20 ml)
Glycerol (or glycerine)	40 ml
Distilled water	20 ml
Cotton blue	0.05 g
(or 1% aqueous solution	2 ml

Preparation :

a. Dissolve phenol in the lactic acid, glycerol, and water by gently heating (if crystals are used).

b. Then add cotton blue.

c. Mix well.

6. Michaelis' Veronal Acetate Buffer (pH range 2.62 to 9.16)

(Raphael, 1983)

Stock Solution A.

Sodium acetate ($C_2H_3NaO_2 \cdot 3H_2O$), 19.428 g (11.704 g of anhydrous salt), and 29.428 g Veronal (Sodium diethylbarbiturate) dissolved in and made to 1000 ml with distilled water.