Studies on Mode of Action and Bio-efficacy of Fungi pathogenic to Larvae of *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* (Linnaeus).

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DOCTOR OF PHILOSOPHY IN ZOOLOGY

By

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This is certified that the corrections suggested by the external referees have been incorporated in the thesis

07/10/11
Dr T B Nayak

07/10/11
(Ashwini Kumar)
Guide

March 2011

This thesis is dedicated to my father

Late Dr. Shyambiharilal Kulashreshtha

&

my mother Mrs. Susheela Kulashreshtha.

DECLARATION

I hereby declare that the Ph. D thesis in the subject of Zoology entitled **"Studies On Mode of Action and Bio-efficacy of Fungi Pathogenic to Larvae of *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* (Linnaeus)"** submitted to Goa University (GU) represents my own work, except where due acknowledgement is made, carried out by me at National Institute of Malaria Research (NIMR), Panaji, Goa and Mycolab, Botany dept., GU, Taleigao under the supervision of Dr. Ashwani Kumar, Deputy Director (NIMR) and Prof. Darbhe Jayarama Bhat, Department of Botany, GU. The thesis has not formed previously the basis for award of degree, diploma or other similar qualifications/ titles submitted to this University or to any other institution.

24/3/11

(Deeparani K. Prabhu)

CERTIFICATE

We certify that the thesis entitled **"Studies on Mode of Action and Bio-efficacy of Fungi Pathogenic to Larvae of *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* (Linnaeus)"** submitted by Mrs. Deeparani K. Prabhu is a record of research work done by her during the period Jan 2003-March 2011 when she worked under our supervision. The thesis has not formed previously the basis for award of degree, diploma or other similar qualifications/ titles submitted to this University or to any other institution.

We affirm that the thesis submitted by Mrs. Deeparani K. Prabhu incorporates the independent work carried out by her under our supervision.

Guide: 24/3/11

(Dr. Ashwani Kumar)

Co-guide: 25/3/2011

(Prof. D. J. Bhat)

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I hope this modest attempt by me of a scholarly pursuit to gain insight on such an important topic will blossom into a valued academic resource.

Mrs. Deeparani K. Prabhu

LIST OF ABBREVIATIONS

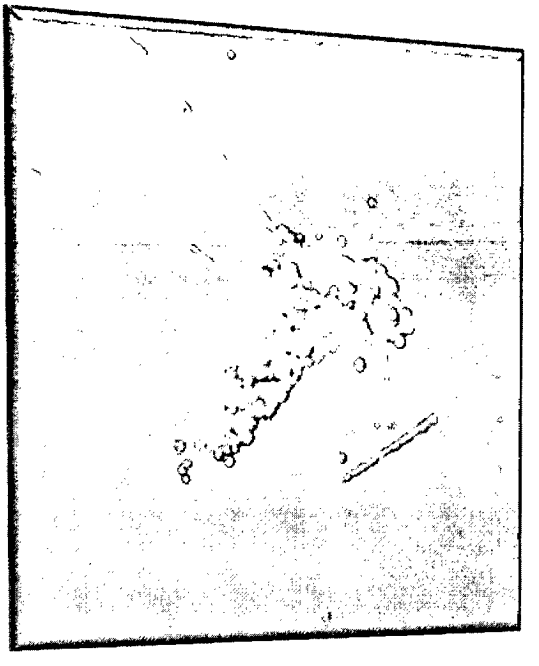
@	at
ARI	Agarkar Research Institute
°C	degree Celsius
$CHCl_3$	Chloroform
CMA	Cornmeal Agar
cm	centimeter
d	days
δ	delta
DHF	Dengue Haemorrhagic Fever
DDT	Dichloro-Diphenyl-Trichloroethane
DMSO	dimethylsulfoxide
DTX	destruxins
et al.	Latin *et alia,* for "and others."
EtOAc	ethyl acetate
e. g	example given
epf	entomopathogenic fungi
GUFCC	Goa University Fungal Culture Collection
h	hour
i.e	that is
IR	infrared
LC_{50}	Lethal concentration of sample required to kill 50% larvae
MCzA	Malt Czapek Agar
MEA	Malt Extract Agar
MeOH	methanol
m. p	melting point
min	minute
mg	milligram
ml	millilitre
μ	micro
NCAOR	National Centre for Antarctic and Ocean Research
NIMR	National Institute of Malaria Research

NMR	nuclear magnetic resonance
NTO	non-target organisms
NaClO	sodium hypochlorite
nm	nanometer
No.	number
O. D	optical density
%	percent
PDA	Potato Dextrose Agar
pH	potential of hydrogen, a measure of acidity/alkalinity of a solution
Pet. ether	petroleum ether
pm	peritrophic membrane
ppm	parts per million
psi	pounds per square inch
RF	retardation factor
RH	relative humidity
rpm	rounds per minute, revolutions per minute
SDA	Saboraud Dextrose Agar
SEARO	South East Asian Regional Office
SEM	Scanning Electron Microscope
sec	second (s)
sp.	species
spp.	species (plural)
TCA	Trichloro acetic acid
TLC	thin-layer chromatography
t.t	test tube
UV	ultraviolet
vs.	versus
WHO	World Health Organization
WNV	West Nile Virus
ZSI	Zoological Survey India

CONTENTS

Chapter 4 Results

Chapter 5 Discussion



Chapter 1

INTRODUCTION

1.1 Mosquito as a Vector:

Mosquito-borne diseases such as Malaria, Filaria, Yellow Fever, Dengue, Chikunguniya, Japanese encephalitis, etc. cause extensive morbidity and mortality, globally. In India there are estimated 29 million cases of filariasis and about 1.5 million reported cases of malaria annually (http://www.searo.who.int/EN/Section313). Mosquitoes are classified in the family Culicidae within the order Diptera. Some 3,490 species are currently formally recognized (Harbach & Howard, 2007) under 44 genera. Female mosquitoes of fewer than 150 species, largely confined to the genera of *Anopheles, Aedes, Armigeres, Coquillettidia, Culex, Culiseta, Haemagogus* and *Mansonia* are vectors of causative organisms of these diseases.

Anopheles stephensi a sub-tropical species, distributed throughout the Middle East and South Asia region, is a primary vector of malaria in urban areas and breeds mainly in man-made breeding sites containing freshwater (Kumar *et al.*, 2007). *Culex quinquefasciatus* on the other hand breeds in organically rich polluted waters and is the principal vector of *Wuchereria bancrofti*, the causative organism of Bancroftian filariasis (Curtis & Feachem, 1981). Dengue is a viral disease transmitted by the domestic container breeding and anthropophilic mosquito *Aedes aegypti* which is its principal vector. Chikunguniya and yellow fever is also vectored *inter alia* by *Aedes aegypti*.

1.2 Mosquito Biology:

Mosquito adults are slender, long-legged, two winged insects easily recognized by their long proboscis and the presence of scales on most body parts. The hindwings have developed into a pair of halteres, small knobbed structures that function as vibrating structure helping in balancing.

Both male and female mosquitoes feed on nectar and only female sucks blood to obtain supplemental nutrition (proteins and iron) to develop eggs.

Larvae are distinguished from other aquatic insects by the absence of legs, the presence of a distinct head bearing mouth brushes and antennae, a bulbous thorax that is wider than the head and abdomen, posterior anal papillae and either a pair of respiratory openings (subfamily Anophelinae) or an elongate siphon (subfamily Culicinae) borne near the end of the abdomen. The immature stages of mosquitoes occupy a spectrum of aquatic environments like temporary or permanent bodies of ground water, leaf axils, tree-holes, rock-holes, crab-holes, bamboo internodes, bromeliads and aroids, fruit shells and husks, fallen leaves, spathes, flower bracts, snail shells, pitcher plants, and artificial containers.

1.3 Control of Mosquito Vector:

Control of mosquito-borne diseases is possible by elimination of the causative organism or control of their mosquito vectors. As affordable solution based on inexpensive vaccines is currently unavailable for protection from most of these diseases, treatment of infected individuals and effective control of vectors is the mainstay of mosquito-borne disease control programmes. Many vector control strategies are available for both adults and larvae. Rapid development of resistance to insecticides have made search for bio-control imperative.

1.4 Bio-control of vectors:

Biological control by harnessing natural enemies and entomopathogens is of interest because of their target precision, handler safety, ecological safety and host specificity. Biological agents such as fishes, bugs, mesocyclops, bacteria (Kumar *et al.*, 1994,

1996; Becker & Ascher, 1998), protozoa (Chapman, 1974; Legner, 1995), nematodes (Kaya & Gaugler, 1993) and fungi (Scholte *et al.*, 2004) have been used as arsenal for mosquito control. Vector control in rice fields was achieved by using the oomycete *Lagenidium giganteum* (Hallmon, *et al.*, 2000). The commercial success of some entomopathogenic fungi in pest control/integrated vector control makes them an attractive option for vector control (Butt *et al.*, 2001).

1.5 Fungi as bio-control agents:

Amongst entomopathogenic fungi, Hyphomycetes have been found to be effective as bio-control agents (Ferron, 1978; Tanada & Kaya, 1993; Hajek & St. Leger, 1994; Wraight & Carruthers, 1999; Keshava Prasad & Bhat, 2007; Mohanty & Prakash, 2010). A recent report focuses on the use of entomopathogenic fungi for mosquito control (Scholte, *et al.*, 2004). Out of the 90 fungal genera reported to be pathogenic to insects and mites, more than 20 genera are reported to be mosquito-pathogenic, prominent amongst them are *Leptolegnia*, *Pythium*, *Lagenidium*, *Crypticola*, *Coelomomyces*, *Conidiobolus*, *Entomophthora*, *Erynia*, *Smittium*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Paecilomyces*, *Penicillium*, *Aspergillus*, *Trichophyton*, *Verticillium*, *Fusarium*, *Chrysosporium* and *Tolypocladium*.

Many entomopathogenic fungi produce insecticidal toxins in liquid culture (Charnley, 2003). Tolypin from *Tolypocladium niveum* was reported to be larvicidal to the larvae of mosquitoes and blackflies (Matha *et al.*, 1988). In the *Culex pipiens autogenicus* larvae the depsipeptide beauvericin from *Beauveria bassiana* caused ultrastructural changes in the midgut epithelium and mortality (Zizka & Weiser, 1993). Extracellular fungal metabolites active against mosquito larvae have been reported from India (Vijayan & Balraman, 1991; Mohanty & Prakash, 2004; Govindarajan *et al.*, 2005; Mohanty *et al.*, 2008b; Soni & Prakash 2010). It

is important to know the action of fungal secondary metabolites on insects if they are to be used effectively in vector control programmes. A successful fungal vector control agent apart from killing selectively mosquitoes and no other organisms, is effective over a large range of salinities, temperatures, relative humidities and breeding sites with variable water quality, is easily and cost-effectively mass-produced and formulated, retains prolonged activity during storage (long shelf life), shows residual activity and persistence in the mosquito population after introduction and is not harmful to humans and other non-target organisms. None of the mosquito-pathogenic fungi presently known exhibit all these characteristics, but they all exhibit at least some.

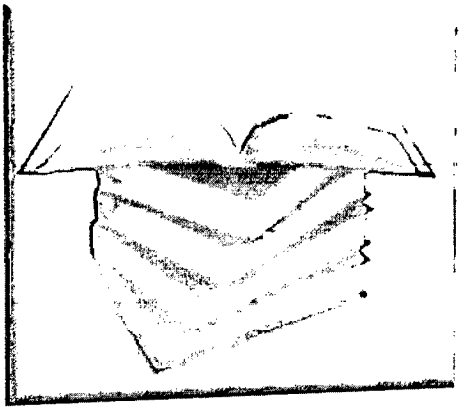
1.6 Present Work:

It was felt that there is a need to isolate, identify and use the promising indigenous mosquito-pathogenic fungal strains and also to gain an insight into pathogenicity, virulence, invasive process, active principles and bio-safety of promising fungi to non- target organisms. Hence, the present work was taken up with the following objectives:

1. Isolation of entomopathogenic fungi; sourcing and maintaining mosquito-pathogenic fungal isolates from the Fungal Culture Collection of National Institute of Malaria Research, Campal, Panaji, Goa and Botany Dept. of Goa University, Taleigao Plateau, Goa.
2. Preliminary bioassay of a few known fungal isolates against laboratory reared mosquito larvae of the three vector species.
3. Studies on mode of action of the fungus to study the invasive and pathological process involved at the histological level of the host.

4. Bioassay of promising isolates against laboratory reared 3rd instar larvae of *Anopheles stephensi* Liston, *Culex quinquefasciatus* Say and *Aedes aegypti* (Linnaeus).
5. Bioassay of crude metabolites of highly effective isolates against vector species with an attempt to separate active fraction/s.
6. Identification of enzymes released by the effective fungal isolates and qualitative analysis of the enzymes (1 or 2 most efficacious isolates).
7. Bio-safety studies of efficacious fungi against non-target organisms using fish (*Aplocheilus blocki* or *Lebistes reticulatus*) and insect (*Notonectid* ug/aquatic/semi-aquatic) models.

The thesis has been organized into 6 Chapters. Besides a brief introduction in this chapter, a review of work done so far on mosquito vectors and biocontrol, isolation and mode of action of entomopathogenic fungi, enzymes, toxins produced by them, biology of mosquito-pathogenic fungi, epizootology of fungal diseases in insects, factors affecting infectivity, host response to fungal invasion, application of the mycoinsecticides and their persistence and recycling in the environment, bio-safety of non target organisms and integrated use of mycoinsecticides in vector management is presented in Chapter 2. Materials used and methods followed in the work have been elaborated in Chapter 3. Results are presented in the Chapter 4 of the thesis in several parts. This is followed by discussion in the 5th Chapter. 6th Chapter encompasses a brief summary of the work carried out. A comprehensive list of bibliography is given at the end of the thesis. Research articles published during the tenure of this work are appended to the thesis at the end.



Chapter 2

REVIEW OF LITERATURE

2.1 Mosquito vectors and biocontrol:

The dawn of new millenium has seen an emergence and re-emergence of mosquito-borne diseases globally due to natural and man-made factors. The resurgence has been attributed to demographic changes, unplanned urbanization; societal changes like human encroachment on natural disease foci, modern transportation, containerized shipping; agricultural changes including changes in land use, irrigation systems, deforestation; genetic changes in pathogens leading to increased epidemic potential; changes in public health including lack of effective vector control, deterioration of public health infrastructure to deal with vector-borne diseases, disease surveillance and prevention programs and possible climate change Gubler (2002). Malaria and dengue re-emerged in the 1970s in Asia and the Americas (Reeves, 1972; Bruce-Chwatt, 1979; Groot, 1980). *Aedes albopictus* a dengue vector is now widespread in the US and other regions of the world through exportation of used tires. In the Indian subcontinent and in parts of South America, urban malaria is a major concern. Malaria control is difficult due to drug resistance in the parasite and insecticide resistance in the anopheline mosquito vectors (Krogstad, 1996). Unavailability of vaccines for most of the mosquito-borne diseases make vector control an important tool to curb these diseases. Ecologically safe insecticides, biological control and integrated vector control can be used for interrupting the disease transmission.

2.1.1 Mosquito as a vector:

Amongst the vector-borne diseases, Malaria is the most important. It is caused by protozoans of the genus *Plasmodium* and is transmitted by the female Anopheline mosquitoes to humans. In the world there are 444 species of *Anopheles* (Harbach, 2004) around 60 species are vectors of the malarial parasites. A species can become a vector when it is susceptible to malaria sporogony, is haematophagic and has enough

longevity to become infective to humans. Many of the species also vector nematodal and viral diseases. In tropical Africa the most efficient vectors of malaria belong to the *An. gambiae* complex, also important is *An. funestus*. In Asia important vectors are *An. culicifacies*, *An. dirus, An. sinensis* and *An. miminus*. A major role is played by *An. farauti* and *An. maculatus* in the Pacific region. In South America the main vector is *An. albimanus*. Closer home in India, 58 *Anopheles* species have been described, of these *Anopheles culicifacies, An. dirus, An. fluviatilis, An. minimus, An. sundaicus* and *An. stephensi* are implicated to be primary malaria vectors (Dash *et al.*, 2007). Amongst these six species except *An. stephensi*, the remaining are species complexes (Sharma, 1998). *An. annularis, An. varuna, An. jeyporiensis* and *An. philippinensis* are vectors of secondary importance (Rao, 1984).

Historically a Sanskrit medical treatise of the 6th century BC Sushruta Samhita describes the symptoms of malarial fever and attributes it to the bites of certain insects. The "intermittent fevers" described in the second century A.D., pointed to the fact that three species of parasites *Plasmodium falciparum, P. ovale*, and *P. vivax* were present (Jones, 1907; Lamb, 1995). In 1476 the term malaria, derived from 'mal''aria' (bad air in Medieval Italian) was probably used first time by Leonardo Bruni in a publication. The value of cinchona bark in treating fevers was known to Peruvian Indians before 1500 A.D. The medicine from the bark is now known as the antimalarial, quinine. In China, Qinghaosu (Artemisinin) was used as a treatment for malaria in 1518–1593 A.D. William Shakespeare born in 1564 at the start of "Little Ice Age" has mentioned the disease 'ague' (in Great Britain malaria was referred as ague) in eight of his plays. In 1880, the malaria parasite was identified in human blood by Charles Laveran and the role of mosquitoes as a vector was established in

1897 by Sir Ronald Ross. Based on this knowledge extensive malaria control programs were carried out after 1900.

There are four species of *Plasmodium causing human malaria P. vivax, P. malariae, P. ovale,* and *P. falciparum*. Currently *P. falciparum* malaria is distributed in the tropics (Snow *et al.*, 2005) while *P. vivax* and *P. malariae* are found in subtropical areas (Hay *et al.*, 2004). The high malaria mortality rate in sub-Saharan Africa *is due to P. falciparum.* As long as the climatic conditions are conducive for the growth and development of anopheline mosquitoes and the development of the parasite, malaria will persist naturally (Takken *et al.*, 2007).

Lymphatic Filariasis (LF), known as Elephantiasis caused by the nematode parasites *Wuchereria bancrofti, Brugia malayi* and *B. timori,* is a major public health problem in tropical and subtropical areas of Asia, Africa, the western Pacific and some parts of the Americas. *W. bancrofti* is responsible for 91% of the estimated prevailing 128 million cases of lymphatic filariasis (Michael & Bundy, 1997). *Culex quinquefasciatus,* known as the tropical house mosquito transmits majority of *W. bancrofti* in the world but in 36 African countries where *W. bancrofti* is endemic, the transmission is mostly by one or two species of *Anopheles*. Several species of *Ochlerotatus* are the main vectors of *W. bancrofti* in Philippines, Myanmar, Thailand and Nicobar Islands of India. *B. malayi* is also vectored by several anophelines and mosquitoes from the genera *Mansonia* in South-east Asia and Indonesia. *An. barbirostris* is the only known vector of *B. timori*. It is endemic in India. There are 29 million filariasis cases in the country and 22 million micro-filaria carriers.

Today in the tropics dengue is the most prevalent mosquito-borne viral disease of humans with estimates indicating 50–100 million cases per year, with 250,000–500,000 cases of dengue hemorrhagic fever (Gubler, 1997). Vectors responsible for

transmission to humans are *Aedes aegypti* and *Ae. albopictus*. Dengue-like illness with hemorrhagic manifestations has been described from China about 1000 years ago (Gubler, 1997). In Asia, Africa, and North America, the first reported epidemics of Dengue Fever occurred in 1779-1780. After World War II a pandemic of dengue began in Southeast Asia and has spread around the globe emerging in the Pacific region and the Americas. In the 1950s, epidemic DHF occurred in Southeast Asia. DHF further expanded in the 1980s when Sri Lanka, India and the Maldive Islands had their first major DHF epidemics. 1980s also saw a re-emergence of epidemic dengue fever after an absence of 35 years, in both Taiwan and the People's Republic of China. In 1994 Pakistan reported an epidemic of dengue fever. The epidemics in Sri Lanka and India were associated with multiple dengue virus serotypes, but DEN-3 was predominant and was genetically distinct from DEN-3 viruses previously isolated from infected persons in those countries.

All the diseases vectored by mosquitoes have been summarized in Table 1.

2.1.2 Disease burden:

Several mosquito-borne diseases in South East Asian (SEA) Region have influenced human health and economy. Except Maldives malaria is endemic in all SEA countries with more than 1, 00,000 deaths reported annually. Altogether 38% of the number of clinical cases in the world is accounted from Asia. The threat of malaria is enhanced by the recent reports of emergence of "tolerant" *P. falciparum* on the Thailand-Cambodia border to artemisinin-based combination therapy (ACT) which is recent and the most efficacious therapy. Lymphatic filariasis (LF) is also a major public health problem in the SEA Region. Nine out of 11 countries are endemic for filariasis with an estimated population of 700 million people. 64% of the global population is

Table 1: Causative organisms, vertebrate hosts and vector genera/species of important diseases transmitted by mosquitoes.

S. No.	Causative Organism	Vertebrate Hosts	Vector Genera/ Species	Disease
1.	Protozoa (*Plasmodium vivax, P. falciparum, P. ovale* & *P. malariae*)	Humans	*Anopheles* spp. (about 60)	Malaria
2.	Viruses Chikungunya virus Group IV Alphavirus (family Togaviridae)	Humans, Monkeys	*Aedes aegypti, Ae. albopictus*	Chikungunya
	DEN group serotypes 1,2,3,4 Group IV (family Flaviviridae)	Humans	*Ae. aegypti, Ae. albopictus*	Dengue
	Yellow fever virus Group IV (family Flaviviridae)	Humans, Monkeys, other animals	*Aedes* spp., *Haemagogus* sp.	Yellow fever
	O'nyong'nyong fever virus Group IV Alphavirus (family Togaviridae)	Humans	*Anopheles* spp.	O'nyong'nyong fever
	Batai virus (family Bunyaviridae)	Humans, Pigs, Birds Ruminants	*Anopheles* spp.	Influenza-like illness
	Cache Valley Virus	Humans,	*Ae. triseriatus, Ae. aegypti, Cx.*	Encephalitis

		Mammals	*pipiens*	
	California Encephalitis virus Gp. V (family Bunyaviridae)	Humans, Rodents, Rabbits, Chipmunks, Squirrels	*Ae. dorsalis, Ae. melanimon*	Encephalitis
	Eastern Equine Encephalitis Virus Group IV Alphavirus (family Togaviridae)	Humans, Horses, Birds, Reptiles, Mammals	*Aedes & Culex* spp., *Culiseta melanurus, Coquillettidia perturbans*	Encephalitis
	Inkoo virus	Humans, Mountain Hare	*Aedes* spp.	Influenza-like illness
	Japanese Encephalitis virus Group IV Flavivrus (family Flaviviridae)	Humans, Pigs, Birds Horses, Cattle,	*Culex tritaeniorhynchus*	Japanese Encephalitis
	Jamestown Canyon Virus	Humans, Deer	*Aedes* spp.	Encephalitis, Meningitis
	La Crosse virus (LACV) Group V (family Bunyaviridae)	Humans, Chipmunks, Squirrels	*Aedes triseriatus*	La Crosse encephalitis
	Murray Valley Encephalitis virus Group IV Flavivrus (family Flaviviridae)	Humans, Birds	*Culex annulirostris*	Australian Encephalitis /Murray Valley Encephalitis
	Rift Valley Fever virus GroupV	Livestock	*Aedes* spp., *Culex* spp.	Rift Valley Fever

	Phlebovirus (family Bunyaviridae)			
	Sindbis virus Group IV Alphavirus (family Togaviridae)	**Humans, Birds, Rodents, Amphibians**	***Aedes* spp., *Culex* spp., *Culiseta morsitans, Mansonia richiardii, Anopheles hyrcanus***	**Karelian Fever**
	St. Louis virus Gp. B Group IV Flavivrus (family Flaviviridae)	**Humans, Birds**	***Culex* spp.,**	**St. Louis Encephalitis**
	Ťahyňa virus (family Bunyaviridae)	**Humans, Hare Hedgehog, Rodents**	***Aedes* spp., *Culiseta annulata, Culex modestus, Anopheles hyrcanus***	**Valtice fever**
	Western Equine Encephalitis Virus Group IV Alphavirus (family Togaviridae)	**Humans, Horses, Birds**	***Culex tarsalis, Culiseta* spp.**	**Western Equine Encephalitis**
	West Nile virus Group IV Flavivrus (family Flaviviridae)	**Humans, Horses, Birds**	***Aedes* spp., *Anopheles* spp., *Coquillettidia perturbans, Culex* spp., *Culiseta* spp., *Deinocerites cancer, Mansonia titillans, Orthopodomyia signifera, Psorophora* spp.,**	**West Nile Encephalitis**
3.	**<u>Nematodes</u> *(Wuchereria bancrofti, Brugia timori & B. malayi)***	**Humans**	***Aedes* spp., *Anopheles* spp., *Culex* spp., *Mansonia* spp. and *Ochlerotatus* spp.**	**Filariasis**

at risk and about 50% of the global figure i.e. 60 million people are either harbouring microfilaraemia or suffering from clinical manifestations (WHO/SEARO, 2006). A loss of US$ 1 billion per year is estimated in India alone on account of LF (Ramaiah *et al.*, 2000).

About 70% people are at risk of dengue globally accounting for 1.8 billion of them residing in the countries of the Asia-Pacific region. In the past five years annually more than 2, 00,000 cases were reported. Major outbreaks of Chikungunya were reported in India, Sri Lanka and Maldives recently. As Dengue and Chikungunya share certain common features they tend to cluster geographically and overlap. Japanese Encephalitis (JE) is on the rise in India, Nepal, Sri Lanka and Thailand with epidemics peaking every three to five years. The estimated annual incidence of JE was 1, 75,000 (Tsai, 2000) and the global burden estimated in 2002 was 7, 09,000 disability-adjusted life years lost (WHO, 2004). JE is rare in Japan due to JE-virus vaccination, use of agricultural pesticides and controlled pig farming. High political commitment, multisectoral collaboration, vector control strategies and community participation are necessary to prevent the emergence of new vector-borne diseases and the re-emergence of those already under control.

2.1.3 The test mosquito species:

Anopheles stephensi a sub-tropical species distributed in the Middle East and South Asia region is considered an important vector of malaria in India, Pakistan and Iran. *An. stephensi* has two races distinguished on the basis of differences of eggs (Rao *et al.*, 1938). The type form, *Anopheles stephensi stephensi,* was reported to inhabit urban areas and the variety *Anopheles stephensi mysorensis* rural areas and generally both have been described to be zoophilic, preferring cattle in rural areas and humans in urban areas. Favorable breeding sites of *An. stephensi* are wells, cisterns, empty

containers, roof, gutters, hoof prints of animals, rice fields, fountains, etc. Larval breeding is significantly higher during the rainy season. Females lay about 100 eggs during one oviposition and maximum nine ovipositions are found to occur. *An. stephensi* is a disease vector of rodent malaria (*Plasmodium berghei* and *P. yoelli*), simian malaria (*P. cynomolgi*) and human malaria (*P. falciparum* and *P. vivax*). It contributes 12% of total malaria cases in India (Adak *et al.*, 2005). Widespread resistance to DDT, HCH and malathion is shown by *An. stephensi* in India (Dash *et al.*, 2008).

Culex quinquefasciatus is widespread in its distribution inhabiting sub tropical Americas, the Afrotropics (White, 1975), Indomalayan, Australasian (Lee *et al.*, 1989), East Asian regions (Bram, 1967), United Kingdom and parts of Middle East. In many urban areas it often comes at night to bite and is a domestic pest (Holder *et al.*, 1999). It is a primary vector of *Wuchereria bancrofti* and vectors avian malaria. In the laboratory West Nile Virus (WNV) is also vectored by it in birds (Sardelis *et al.*, 2001) and it was found to be infected in nature by WNV in the Florida keys (Hribar *et al.*, 2004). Western equine encephalomyelitis and St. Louis encephalitis have been isolated from this species and it has been implicated as a vector of dog heartworm (Carpenter & LaCasse, 1955, Sirivanakarn, 1976). It was found to be infected with Chikungunya virus during an epidemic that occurred in 1953 in southern Tanzania (White, 1971). More recently in Senegal, Wills *et al.* (1976) demonstrated the presence of hepatitis B virus on the mouthparts of these mosquitoes. Reovirus type 3 can be picked up by *Cx. quinquefasciatus* larvae and it is maintained through the pupal stage to the first days of adult life. It could also be an important vector of Rift Valley fever virus, (WHO, 1978). It can be one of the vectors of West Nile virus in India (Rao, 1975), and it can transmit St. Louis encephalitis virus in the Americas

(Gillett, 1972). In Australia it is the main vector of fowl pox virus and is of importance in veterinary medicine (Lee *et al.,* 1958). It can also transmit the yellow fever virus in the laboratory (Muspratt, 1956) but has no role in the natural transmission of yellow fever. In lab it is able to transmit smallpox virus, which it could hold for about 72 h (Sarkar *et al.,* 1973). Similarly it can transmit Wesselbron virus (Simasathien & Olson, 1973) and Venezuelan equine encephalitis virus (Kramer & Scherer, 1976). *Cx. quinquefasciatus* which bites only in darkness is responsible for transmitting 98% of LF parasite in the endemic regions of urban India. *Cx. quinquefasciatus* typically breeds in stagnant, organically polluted water (Sunish *et al.*, 2008). Flooded pit latrines and soakage pits being the major breeding sites of *Culex* in the urban scenario, can be marked for specific control measures (Curtis & Feachem, 1981).

Aedes (Stegomyia) aegypti (Linnaeus, 1762) (*Diptera, Culicidae*), is one of the most widespread mosquitoes and the principal vector of dengue and yellow fever (*Flaviviridae*) as well as Chikunguniya (*Togaviridae*) viruses (White, 2003). It is a potential vector of dog heartworm, and Murray Valley encephalitis and Ross River viruses. Variations were observed in the vector competence of the *Ae.aegypti* populations (Gubler *et al.*, 1979). Two morphological and behavioral forms recognized are *Ae. aegypti aegypti* the more urban form present in the New World, Asia, and Pacific and *Ae. aegypti formosus* which originates from African tropical forests with both domestic and sylvatic populations. It oviposits in any type of man made containers or storage containers having even a small quantity of water. The eggs can resist desiccation for up to 1 year. This species is active in the day and prefers to bite indoors or in the shade. Special problems confront the control of aedine vectors due to their inaccessible breeding sites and aggressive biting behaviour.

2.1.4 Vector control methods:

Vector control is an important part of control/eradication of mosquito-borne diseases. In the USA, USSR, southern Europe and most Caribbean Islands malaria eradication was achieved mainly by vector control in 1940-1960. Throughout history the most critical factor in the spread or eradication of disease has been human behavior (shifting population centers, changing farming methods and the like). The yellow fever virus travelled from Africa to the Americas in a slave ship. And the Asian tiger mosquito, a particularly aggressive creature, was introduced in Texas through the used tyres imported from Japan and Taiwan. Vector control in India prior to 1936 was achieved by provision of proper drainage system and targetting the *Anopheline* breeding habitats to eliminate the larvae by using larvivorous fishes, oils, Paris green and by use of pyrethrum extract space spray to eliminate adults.

Current mosquito vector control strategies involve a number of different tactics including physical, chemical, biological control methods, community participation, environmental management etc. as tabulated in Table 2.

Larval Control:

A. Physical control: Source reduction – Minor and major engineering methods like surface leveling, drainage, filling, flushing, pumping of water, removal of vegetation. Floating layers of expanded polystyrene beads (EPBs) create a physical barrier to egg-laying adult *Culex* while suffocating larvae and pupae. In Zanzibar, Tanzania (Maxwell *et al.,* 1990, 1999), and Tamil Nadu, India (Sunish *et al.*, 2002), application of expanded polystyrene beads (EPBs) in all pits found to be breeding-sites of *Cx. quinquefasciatus* virtually eliminated the *Culex* nuisance mosquito problem. Upgraded sanitation and drainage is undoubtedly the long-term solution to the urban *Culex* problem. The larvae and pupae of the genus *Mansonia* attach their breathing

tubes to underwater roots, stems and leaves of floating aquatic plants. Therefore, removal of host plants by herbicides or mechanically is effective to prevent *Mansonia* production.

B. Chemical control: Larviciding with chemical insecticides like Temephos and Fenthion is in use, in addition to a distillate of crude oil and malaria larvicidal oil.

C. Biological control:

The ever increasing resistance of vectors to chemical insecticides and the concomitant damage to the environment make the biological control of mosquito populations an attractive proposition. Biological Control is defined as "The action of parasites, predators, or pathogens (disease-causing organisms) in maintaining another organism's population density at a lower average than would occur in their absence" (DeBach, 1964). A biological control agent should be selected on the basis of adaptability to the introduced environment, preference for the selected target and overall interaction with the indigenous organisms (Kumar & Hwang, 2006).

The biocontrol agents can be categorized as parasites, predators and pathogens.

Parasites: The protozoan parasite *Ascogregarina* releases sporozoites disrupting the gut wall of mosquito larvae (Beier & Craig, 1985). *Ae. albopictus* and *Ae.*aegypti are infected by *Ascogregarina taiwanensis* and *As. culicis* (Munsterman, 1990). Another mosquito larval parasite evaluated for its biocontrol potential is the mermithid nematode, *Romanomermis iyengari* against genera of *Aedes, Anopheles* and *Culex* mosquitoes in field and laboratory experiments (Chandrahas & Rajagopalan, 1979; Santamarina *et al.*, 1996).

Predators: Most aquatic insects predate on mosquito larvae and pupae as natural food (Ellis & Borden, 1970; Peckarsky, 1984). The backswimmer, *Notonecta undulate* (Hemiptera, Notonectidae), preys upon mosquito larvae (Murdoch *et al.*, 1984;

Blaustein, 1998). The backswimmer, *Anisops assimilis*, was known to control mosquitoes since 1939. The efficiency of backswimmers to predate on mosquito larvae was found to be container-specific (Lester & Pike, 2003). The oviposition rates by adult mosquitoes in a water body showed decline due to the presence of backswimmers (Chesson, 1984; Blaustein *et al.*, 1995).

Toxorhynchites, a kind of mosquito with cannibalistic larvae, has been used as biological control agent. For the control of *Ae. aegypti* certain kinds of *Toxorhynchites* mosquitoes are good because they breed in the same kinds of containers. However, it proved to be unsuccessful in the field. The adult dragonflies and damselflies are efficient predators of adult mosquitoes while their larvae utilize mosquito larvae as food.

In the developed and developing countries biological control, using larvivorous fish, was important to malaria control programmes in the 20th century, particularly in urban and periurban areas. The introduction of mosquito fish *Gambusia* and *Aplochelius* for biocontrol was carried out all over the world extensively. However *Gambusia* introduced into different water bodies around the world was shown to produce important impacts on the native biological communities (Kumar & Hwang, 2006). In 1908, the guppy *Poecilia reticulata*, from South America, was introduced for malaria control into British India and many other countries (Gerberich, 1985). *Gambusia* is most efficient in clean water, while *Poecilia* can be used successfully in organically polluted water. The annual killifishes, *Cynolebias*, *Nothobranchius* and *Aphyosemion*, have drought-resistant eggs and could be used in breeding sites that temporarily dry out, such as borrow-pits and irrigated rice fields. In some rice fields of Asia, Grass Carp (*Ctenopharyngodon idella*) has been used which controls mosquito larvae and

Table 2: Vector control strategies using various products.

Vector Control Method	Intervention	Target	Products
Community education	Behavioral change, application of all other interventions	All mosquito vectors	
Environmental management and sanitation	Natural environment changes	Lymphatic Filariasis (LF) vector	polystyrene beads in standing water bodies
	Improved housing quality	Vectors of Malaria, Dengue	
	Physical barriers to breeding sites	Vectors of LF	
Biological control	Larvivorous fishes Larviciding	All mosquito vectors Urban mosquitoes	*Gambusia* sp., *Poecilia* sp., *Aplocheilus* sp., etc., microbial larvicides, Bacilli- *Bacillus thuringiensis* var. *israelensis* (*Bti*), *B. sphaericus* (*Bs*); Fungi- *Lagenidium* sp.

Chemical control	Space Spraying	Urban mosquitoes	pyrethroids, organophosphates
	Indoor Residual Spraying	Vectors of Malaria, LF	pyrethroids, organophosphates, carbamates, DDT (malaria only)
	Insecticide-treated materials (ITNs, Curtains, etc.)	Vectors of malaria, LF	pyrethroids
	Household products	All mosquitoes	aerosols, coils, mats, repellents, natural products, etc.

provides a crop of edible fish. In domestic water containers larvae have been eliminated by using small fish, such as *Clarias fuscus*, *Tilapia nilotica*, and *Macropodus* sp. (Neng *et al.*, 1987).

Omnivorous tadpoles are potential predators of mosquito larvae. Aquatic insects growing in water containers are destroyed by larvae of the giant Cuban tree frog *Hyla septentrionalis* (Webb & Joss, 1997; Komak & Crossland, 2000).

Pathogens: In India two biolarvicides tested were *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*), resistance to *Bs* developed soon after its application (Adak, 1995). Presently *Bti* is used in public health programmes as anti-larval measure in urban areas. The microbial product composed of a dormant spore form of the bacterium and an associated toxin is ingested by the mosquito larvae. In the insect the toxin binds to the receptor cells in the stomach and acts as a poison but not in mammals (Lacey & Undeen, 1986; Bishop *et al.*, 1999; Batra *et al.*, 2000). The application of biocide destroys mosquito larvae but does not harm the predators present in the water (Ohana *et al.*, 1987; Su & Mulla, 2005).

Three genera of fungi *Lagenidium*, *Coelomomyces*, and *Culicinomyces* (Roberts, 1974; Lacey & Undeen, 1986; Federici, 1995) pathogenic to mosquito larvae are important biocontrol agents. The oospores of the fungus *Lagenidium giganteum* have the ability to control mosquito populations. They resist drought, can be produced in bulk, survive for many years in the soil, but are reactivated around a month after flooding. The spores are activated by moistening for 1-2 week before being sprayed on the site to be treated (Copping & Menn, 2001). *Coelomomyces* spp. have narrow host ranges, cause natural epizootics in the mosquito populations and are found to kill many mosquito species. Recently in the laboratory mortality was seen in the adult

mosquitoes exposed to Hyphomycetes *Beauveria bassiana* (Clark *et al.*, 1968) and *Metarhizium anisopliae* (Scholte *et al.*, 2003b).

From the actinomycete, *Sacchoropolyspora spinosa*, a fermentation product obtained called spinosad, is another naturally derived larvicide (Williams *et al.*, 2003). Like chemical insecticides microbes also can be stored and packed easily. They have similar method of application and the evaluation of treatment effects is performed in same manner as for insecticides. They only differ in the period of application and effective mortality which is usually longer for microbial insecticides.

Adult Control:

A. Indoor residual spray: The identification of insecticidal action of Dichloro-Diphenyl-Trichloroethane (DDT) in 1939 by Paul Mueller revolutionized the mosquito control programme. In the army camps in United States and United Kingdom for the first time DDT was used as an anti-mosquito spray. Malaria saw a sharp decrease from 75 million cases to 0.1 million cases in India in 1966 by using DDT as IRS (Sharma, 2003). Synthetic Pyrethroids (SPs) such as deltamethrin, cyfluthrin and lambdacyhalothrin are used in public health as residual insecticide and for impregnation on mosquito nets. Resistance against SPs has been reported from areas where SPs are in use (Singh *et al.*, 2002). Also resistance to other insecticides like HCH, dieldrin and malathion used on walls of houses has been reported (Sharma & Samnotra, 1962; Rajagopal, 1977).

B. Space spray: Adult control is also carried by spraying of mists, fogs or aerosols in the rooms in the form of suspended particles of insecticides in the air to kill the flying mosquitoes generally contact or respiratory poisons eg: hydrocarbons, organophosphorous compounds, carbamates, synthetic pyrethroids.

C. Personal Protection: The most common method of personal protection is use of repellents. Non- ester pyrethroids like permethrin, etofenprox and derivatives of plants like lemon, eucalyptus, neem and citronella have shown adequate protection against mosquito bites. The use of insecticide-treated bednets (ITNs) and curtains for protection against mosquitoes is strongly advocated. A shift from human to animal feeding was observed in Kenya after the use of pyrethroid impregnated bednets (Bogh *et al.*, 1998). In India field trials using hessian curtains impregnated with deltamethrin showed statistically significant reduction in the densities of the mosquitoes *An. subpictus* and *Cx. quinquefasciatus*.

Genetic Control:

Genetic control is achieved by:

1. Inducing sterility in the vector mosquitoes using irradiation or chemosterilants.

2. Introduction of genes which prevent development of malaria parasites in vector mosquitoes by making them non-susceptible to *Plasmodium* (Collins & Paskewitz, 1995) or divert them from being strongly attracted to biting humans.

Integrated Vector Control:

Since 2001 the World Health Organization has been promoting Integrated Vector Management (IVM) as the new strategic approach to vector control. It incorporates environmental management and biological control methods, role of insecticides is bare minimum and is highly judicious. IVM is based on the concept that for effective control the health sector requires the collaboration of various public and private agencies and the community participation. Secondly, IVM emphasizes capacity building at the district and municipal level to plan, implement, monitor and evaluate these vector control operations.

Dependence of mosquito control on a limited number of tools, in particular pyrethroids, which could be lost to resistance at any time, makes research and development to deliver a steady output of replacements imperative. Novel ecofriendly biopesticides in combination with Integrated Vector control programmes can help in reducing the problem substantially.

2.1.5 Fungi as biocontrol agents:

Fungi comprise a large diverse group of eukaryotes with absorptive nutrition growing on a variety of substrates. They have distinct cell wall, are achlorophyllous, unicellular (yeasts) or multicellular with filamentous hyphae forming a mycelium, the latter being characteristic for most of the fungi attacking insects and reproducing by sexual or asexual, spores. They are best adapted to wet or moist habitats and are usually easily cultured on artificial media.

The group of fungi which generally inhabits insects, spiders and mites are called entomogenous (Gr: 'entomon' = insects; 'genesis' = arising in) fungi. The term entomogenous fungi encompasses all types of association between insects and fungi, with disease causing fungi being referred to as 'entomopathogenic fungi' (epf).

Fungi are common pathogens causing insect disease in the field with spectacular epizootics reducing populations of specific insects significantly (Carruthers and Soper, 1987; Butt *et al.*, 2001; Inglis *et al.*, 2001; Lacey *et al.*, 2001). Therefore, there has been great interest in using fungi to control insects. Agostino Bassi, after whom the insect-infecting fungus *Beauveria bassiana* is named, put forth the concept of using microorganisms to control destructive insects in 1836. Louis Pasteur in 1870 with his experimental work on microsporidian Pébrine disease of silkworms gave an insight into the nature of infectitious diseases setting the trend to use pathogens to control pest insects (Pasteur, 1870). In 1879 Elie Metschnikoff after studying the

natural infection of the wheat cockchafer *(Anisoplia austriaca* Herbst*)* by the green-muscardine fungus [*Metarhizium anisopliae* (Metsch.) Sorok.] was successful in artificial propagation of *M. anisopliae* and advocated mass production of epf for controlling insects (Metschnikoff, 1880). Till the beginning of the 20th century fungi dominated the scene of microbial control with extensive studies carried out for the control of scale insects and other citrus insects (Steinhaus, 1956). Currently more than 750 species of insect-infecting fungi have been documented (Roberts & Humber, 1981; Samson *et al.*, 1988; Evans & Hywel-Jones, 1997; Tzean *et al.*, 1997; Faria & Wraight, 2001; Goettel *et a*l. 2000; Lacey *et al.*, 2001). Almost all Entomophthorales, a few yeasts, some Chytrids numerous Ascomycetes and Fungi Imperfecti and the Basidiomycete *Uridinella* are entomopathogenic. The significance of fungi pathogenic to insects as biocontrol agents has been reviewed by Burges (1981), Latge & Moletta (1988), McCoy *et al.* (1988), Roberts (1989), McCoy (1990), Ferron *et al.* (1991), Tanada & Kaya (1993), Hajek & St. Leger (1994), Charnley (1997), Roy *et al.* (2006). Fungi are "contact" pathogens. Life cycles of Entomophthora involve a sexual stage and resting spores, whereas imperfect fungi have a simple life cycle with only asexual phase characterized by production of naked conidia free on the mycelia (Pell *et al.*, 2001).

Successful control of insects with fungi has been achieved. *Verticillium lecanii* was used to control aphids, scale insects, thrips and red spider mite in the glasshouse in UK (Hall, 1981; Gillespie *et al.*, 1982). The hyphomycetous *M. anisopliae* and *B. bassiana* have been tested extensively for use against foliar and soil insects (Roberts & Humber, 1981; Ramoska & Todd, 1985; Butt *et al.*, 1992; McDowell *et al.*, 1990). In field-crop situations *Beauveria* spp. to control Colorado potato beetle and the

codling moth and *Metarhizium* spp. to control spittlebugs have been used successfully all over the world (Ferron, 1981).

Fungal biocontrol agents (BCA) could be harnessed where chemical pesticides like organochlorines have been banned or are being phased out or where vectors have developed resistance to conventional insecticides. Contamination of ground water and effect on food web by chemical insecticides make fungal BCAs an attractive environmentally friendly option. Direct host invasion, enzyme production, physiological starvation, toxin production in insects targeted along with host specificity and safety towards non-target organisms, give fungal BCAs an edge over other agents.

Amongst the alternatives available for mosquito control they are comparatively easy to handle, the spores are easier to produce in bulk in favourable conditions and in unfavourable conditions resting stages are formed making them the BCAs of choice. The increasing number of commercial products available or under development reflects the growing interest in exploring the BCAs for insect control (Table 3).

2.2 Isolation of entomopathogenic fungi:

Prospecting epf fungi resulting in a commercially viable biocide entails a number of steps. The fungal species must be isolated from a diseased insect or from nature and identified. The background to diagnose insect diseases has been given by Steinhaus (1963). Lacey & Brooks (1997) have reviewed the initial handling and diagnosis of diseased insects. Weiser (1977), Poinar & Thomas (1984) and Samson *et al.* (1988) have given atlases which help in distinguishing a diseased insect in the field.

Entomopathogenic hyphomycete fungi are readily recovered from soils from disparate habitats including agricultural and forest systems (Barker & Barker, 1998; Bidochka

Table 3 Commercial scale production of mycoinsecticides			
Fungus	**Target**	**Producer**	**Product**
Verticillium lecanii	Whitefly & Thrips	Koppert, Netherlands	Mycotal
Metarhizium anisopliae	Cockroaches	Eco Science, USA	Bio-Path
"	Vine weevil	Bayer, Germany	BIO 1020
Metarhizium flavoviride	Locusts & Grasshoppers	CABI Bio Science, UK	Green muscle
Beauveria bassiana	Coffee-berry borer	Live Systems Technology, Colombia; AgrEvo, Germany	Conidia
B. bassiana	European corn-borer	Mycotech, USA	Corn Guard
B. bassiana	Colorado beetle	Former USSR	Boverin
Beauveria brongniartii	Cockchafers	Andermatt, Switzerland	Engerling -spilz
Paecilomyces fumosoroseus	Whitefly	Agrobionsa, Mexico	Pae-Sin
"	"	Biobest, Belgium	PreFeRal
Lagenidium giganteum	Mosquito larvae	Agra Quest, USA	Laginex

(Compiled from Charnley 1997; Burges, 1998; Butt & Copping, 2000)

et al., 1998). If the fungus has already sporulated it can be directly isolated from the insect cadaver as seen in most Hyphomycetes (Goettel & Inglis, 1997). The conidia of entomophthoralean fungi can be directly showered on nutrient medium (Papierok & Hajek, 1997). In case of non-sporulating diseased insects the sporulation can be hastened by incubating in a moisture chamber. Live baiting with insects (Zimmerman, 1986), direct extraction using aqueous medium (Beilharz, *et al.*, 1982) or discontinuous density gradients for soil fungi (Hajek & Wheeler, 1994) are some popular isolation techniques. Using various substrates like hemp aquatic fungi can be baited (Kerwin & Peterson, 1997). The growth of saprophytic fungi and bacteria is inhibited by adding fungicide and/or antibiotics to the selective medium. Goettel & Inglis (1997), Humber (1997), Kerwin & Peterson (1997), Papierok & Hajek (1997) have discussed in depth the isolation and storage of entomopathogenic fungi.

2.3 Biology of mosquito-pathogenic fungi:

An attempt to generate an exhaustive list of fungi which were either sourced from mosquitoes or assayed against them in different parts of the world has been carried out in this part of review of literature. The mosquito hosts, infected stage of the host lifecycle, the mosquito-pathogenic fungi, geographic distribution from where they were sourced or assayed either in the lab or field along with references have been tabulated in Table 4 and Table 5.

The family Culicidae has been classified into Subfamily *Anophelinae* and Subfamily *Culicinae* by Harbach & Kitching (1998), the mosquito genera have been arranged following the same and the species organization is in alphabetical order, the abbreviation of genera followed is as recommended by Reinert (2001). The taxonomy and nomenclature of fungi is based on Kirk *et al.* (2001) except that of anamorphic

fungi. The table is further divided based on fungal classes with orders given in brackets. To summarize the data from Table 4 and 5 approximately 21 genera of fungi are pathogenic to Anophelines whereas 28 genera are pathogenic to Culicines (Table 6). From Subfamily *Anophelinae* around 41 species are infected and from Subfamily *Culicinae* almost 115 species are reported to be susceptible with approximately 60 spp. from the genus *Aedes*. Amongst these mosquito-pathogenic fungi only few have been discussed which are either known to cause epizootics or are reported to have potential in vector control.

1. Oomycetes:

The Oomycetes (watermolds) are a class of fungi currently placed under Kingdom Chromista along with diatoms and brown algae (Sleigh, 1989) its history of phylogenetic relationship with fungi is beset with many controversies (Copeland, 1956; Kerwin & Peterson, 1997). A few genera like *Aphanomyces, Leptolegnia, Saprolegnia, Pythium, Crypticola* and *Lagenidium* are facultative parasites of mosquito larvae the latter has reached commercial stage in mosquito control.

Leptolegnia caudata was sourced from *Anopheles culicifacies* (Bisht *et al.*, 1996; Fukuda *et al.*, 1997), while *L. chapmanii* was isolated from *Ochlerotatus triseriatus* (Seymour, 1984). *L. chapmanii* is pathogenic to *Ae. aegypti, Cx. quinquefasciatus*, *Anopheles quadrimaculatus* and *Anopheles albimanus.*

Lagenidium giganteum: *Lagenidium giganteum* is the only species of the genus *Lagenidium* which is a facultative parasite of mosquito larvae. The first strain was isolated from Daphnia in North Carolina (Couch, 1935). It was shown to cause natural epizootics in *Culex territans* in 1975 in Louisiana (Glenn & Chapman, 1978). Compared to Subfamily Anophelinae mosquitoes, it has a wider host range in Subfamily Culicinae. Its geographical distribution spans the temperates being found

in North America, Europe, Africa, Asia, and even Antarctica (Federici, 1981). The activity of fungus is limited by brackish or organically rich aquatic habitats (Merriam & Axtell, 1982) and temperatures lower than 19°C (Suh & Axtell, 1999). Life cycle involves asexual zoospores and sexual oospores. Mosquito larva is invaded through the buccal cavity or the cuticle of the head capsule by the biflagellate motile zoospores. Variation in susceptibility of different instars has been reported with higher susceptibility in the second and third instars of *Ae. aegypti, An. stephensi* and *Cx. pipiens* compared to first and fourth instars (Lord & Roberts, 1987). This has been attributed to the small size of the first and thick cuticle of the fourth instar making it difficult for the zoospore to attach and penetrate.

Aedes notoscriptus, Anopheles farauti, *Culex annulirostris*, *Culex quinquefasciatus* (Frances, 1991) and *Aedes aegypti* (Frances *et al.*, 1989; Frances, 1991) were susceptible to *Crypticola clavulifera* in the laboratory assays carried in Australia.

2. Chytridiomycetes:

Chytridiomycetes are aquatic saprobes or parasites with motile zoospores.

Coelomomyces: The genus *Coelomomyces* comprises of around seventy species. All are obligate parasites and their life cycle shows alternation of generation between the sexual (gametophytic) and asexual (sporophytic) stages (Couch & Bland, 1985). The host range of *Coelomomyces* is mostly restricted to aquatic Dipteran insects, like *Culicidae, Psychodidae, Chironomidae, Simuliidae* and *Tabanidae* (Chapman, 1974; Roberts, 1970). The chytridiomycete *Coelomomyces* and the Oomycete *Lagenidium giganteum* together have been studied from vector control point of view making up the bulk of studies on entompathogenic fungi that affect mosquito larvae.

Coelomomyces spp. require a copepod host to complete their life cycle. The mosquito larval cuticle is penetrated by biflagellate zygotes followed by myceliar growth filling

the haemocoel. Then it produces zoospores which seek the copepod host. Then haploid uniflagellate isogametes are produced which fuse to form diploid zygotes. Sometimes a few infected mosquito larvae pupate and later produce infected adults. In case of adult female *Ae. aegypti* the infection is limited to ovaries (Lucarotti, 1992). Eggs are substituted by the resting spores in the ovipositional attempts made by them (Lucarotti 1987, 1992). The fungus is transmitted to newer habitats where *Aedes* usually breeds like water containers and tree holes (Lucarotti & Andreadis, 1995; Shoulkamy *et al.*, 1997). *Coelomomyces* spp. are known to cause significant epizootics, in larval populations persisting over several years and causing high mortality rates aiding in natural control (Apperson *et al.*, 1992). *Coelomomyces indicus* Iyengar, is a strain which causes periodic epizootics in rice fields in Egypt (Gad & Sadek, 1968), Kenya (Service, 1977a) and Southeast Asia (Whisler *et al.*, 1999). Field trials for vector control have been carried out. To control the filaria vector *Aedes polynesiensis* Marks *Coelomomyces stegomyiae* var. *stegomyiae* was introduced on a Pacific island (Laird, 1967). This trial was successful with the fungus persisting actively in new locality for seven years.

Some are of the opinion that complex life cycle with fluctuating infection rates makes it unsuitable for the control of mosquito populations, (Service, 1983) while others after studies on different coelomomycete species, deduced that the fungus has biocontrol potential (Federici, 1981; Lacey & Undeen, 1986).

3. Zygomycetes: Sexual reproduction through aflagellate zygospores is the characteristic feature of Class Zygomycetes. It includes order Entomophthorales which has 200 known species of EPF spread in six genera (Humber, 1997). Entomophthorales are obligate pathogens infecting wide host range of adult insects with *Entomophthora aquatica* and *Entomophthora conglomerate* infecting aquatic

stages. Certain species have restricted host range while some are mosquito pathogens (Humber, 1997).

Entomophthora: These infect adult rather than larval mosquitoes. From *Culex pipiens* adult *Entomophthora culicis* (Braun) Fresenius was isolated in Germany (Braun, 1855). It infects *Culex* spp., *Oc. detritus*, *An. maculipennis*, *An. cinereus* and *Aedes* species (Kramer, 1982). Mosquitoes from the genera *Aedes*, *Culex*, *Anopheles* and *Culiseta* are infected by *Entomophthora conica*, *E. culicis*, *E. destruens*, *E. gracilis*, *E. henrici*, *E. papilliata, E. radicans*, *E. rhizospora, E. schroeteri*, *E. thaxteriana* and *E. variabilis* (Anderson & Ringo, 1969; Roberts, 1974; Eilenberg, 2000). Most species are unable to grow under mass-production fermentation conditions hence could not be used for mosquito control (Papierok & Hajek, 1997; Eilenberg, 2000). *Conidiobolus coronatus* was found infecting *Cx. quinquefasciatus* from an outdoor colony of adult mosquitoes (Lowe *et al.*, 1968) and has widest host range but it is the only entomophthoralean reported to infect vertebrates ruling out its use in biocontrol (Emmons & Bridges, 1961; Bras *et al.*, 1965).

Trichomycetes: Members of this class are arthropod gut fungi (Lichtwardt *et al.*, 1999). The genus *Smittium* of order Harpellales has mosquito pathogenic species. *Smittium* spp. is reported to cause high mortalities in *An. gambiae* (Coluzzi, 1966), *Ae. aegypti* and *Cx. pipiens molestus* (Dubitskij, 1978) apparently caused by blockage of the rectum. In Argentina larvae of *Aedes, Anopheles*, *Culex*, *Mansonia*, *Psorophora* and *Uranotaenia* species were infected by *Smittium morbosum* (García *et al.*, 1994).

Deueteromycetes (Hyphomycetes): Class Deuteromycetes comprises of filamentous fungi which reproduce by asexual conidia. Majority of them are anamorphs of ascomycetes and they are also referred to as anamorphic fungi or Fungi imperfecti. The most common entomopathogenic genera include *Aspergillus*, *Beauveria*,

Culicinomyces, Fusarium, Hirsutella, Metarhizium, Nomuraea, Paecilomyces, Tolypocladium and *Penicillium.*

Culicinomyces clavosporous Couch, Romney and Rao has received widespread attention It parasitizes a wide range of Dipteran larvae including mosquitoes. *Culicinomyces* lifecycle is asexual with ingestion of conidia adhering and penetrating the foregut or hindgut cuticle. *Culicinomyces clavisporus* infections are limited by high salinity and organic pollution (Sweeney, 1978). No intermediate host is required for the life cycle and large scale production is possible, the only limitation is short shelf life of conidia (Sweeney, 1981; Roberts *et al.*, 1987).

Beauveria bassiana: The hydrophobic conidia of *B. bassiana* were applied in the form of conidial dust on breeding surfaces and infected mosquito larvae (Clark *et al.*, 1968). They infected at the tip of the siphon and head region (Miranpuri & Khachatourians, 1991).

It is pathogenic against the *Ae. aegypti*, *Oc. dorsalis*, *Oc. hexodontus*, *Cx. pipiens*, *Culex tarsalis* and *Cs. incidens* (Pinnock *et al.*, 1973); *Cx. pipiens*, *Cx. tarsalis*, *Cx. tritaeniorhynchus* and *An. albimanus* larvae (Clark *et al.*, 1968; Sandhu *et al.*, 1993; Geetha & Balaraman, 1999). Assays against adult *Cx. tarsalis*, *Cx. pipiens*, *Ae. aegypti*, *Oc. sierrensis*, *Oc. nigromaculis* and *An. albimanus* caused 100% mortality but 50% mortality was seen in control, the route of invasion appeared to be spiracle and release of a toxin was implicated in the death (Clark *et al.*, 1968). Successful laboratory tests against adult *An. gambiae* have been carried by Scholte *et al.*, (2003a, b). *B. bassiana* was found to produce a number of toxins like Beauvericin, Bassianin, Bassianolide, etc. and *B. brongniartii* produced Oosporein (Ferron, 1981; Grove & Pople, 1980; Strasser *et al.*, 2000).

Metarhizium: The larvicidal effect of *Metarhizium anisopliae* conidia were observed in *An. stephensi*, *An. quadrimaculatus*, *Ae. aegypti*, *Oc. atropalpus*, *Oc. taeniorhynchus*, *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius* by Roberts (1970). High susceptibility of larvae in lab, economic large scale production and ease in conidial storage make it an attractive mosquito control agent (Scholte *et al.*, 2004).

Tolypocladium: *Tolypocladium cylindrosporum* Gams was isolated from *Oc. sierrensis* in California in 1971 (Soarés, 1982). It was suggested as a candidate for control of mosquito larvae in temperate areas (Goettel, 1987 a, b) but the fungus was not effective in two different field studies in New Zealand (Gardner & Pillai, 1987) and Canada (Goettel, 1987b).

Penicillium: In Brazil *Penicillium canescens*, *P. chrysogenum*, *P. citrinum*, *P. corylophilum*, *P. decumbens*, *P. expansum*, *P. fellutanum*, *P. implicatum*, *P. janthinellum*, *P. oxalicum*, *P. purpurogenum*, *P. viridicatum* and *P. waksmanii* have been isolated from *Aedes* spp., *Anopheles* spp., *Culex* spp. and *Mansonia* spp. adult and larval mosquito stages with maximum number of species isolated from *Anopheles* spp. (da Costa & Oliviera, 1998). There are reports stating that species such as *P. corylophilum*, *P. waksmanii*, *P. fellutanum* and *P. janthinellum* are mosquito-pathogenic (da Costa, *et al.*, 1998).

2.4 Mode of action of Fungi:

2.4.1 Infectitious propagule:

The ablity of an epf to produce and deliver propagules to infect susceptible hosts determines its success. Generally the spore stage represents the infectitious propagule (Charnley, 1984). *Nomuraea rileyi* a pathogen of lepidopteran larvae produces

Table 4: List of few mosquito species of Subfamily *Anophelinae* on which fungal species were detected or tested either in laboratory or field.

Mosquito Host	Host sta-ge infec-ted	Mosquito-pathogenic fungi [Order] *Species*	Locality	References
OOMYCETES				
[SAPROLEGNIALES]				
Anopheles sp.	L	*Leptolegnia chapmanii*	Argentina	López Lastra *et al.* (2004)
An. albimanus	"	*Aphanomyces* sp.	USA	Seymour & Briggs (1985)
"	"	*Leptolegnia chapmanii*	USA	McInnis & Zattau (1982)
An. culicifacies	"	*Aphanomyces laevis*	India	Bisht *et al.* (1996)
An. culicifacies	"	*Leptolegnia caudata*	"	Bisht *et al.* (1996)
An. gambiae	"	*Leptolegnia* sp.	Uganda	Nnakumusana (1986),
An. maculipennis	"	*Saprolegnia mixta*	USSR	Kalvish & Kukharchuk (1974).
An. quadrimaculatus	"	*Leptolegnia chapmanii*	USA	McInnis & Zattau (1982), Lord & Fukuda (1990)

[PYTHIALES]				
An. freeborni	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
"	L	*Lagenidium giganteum*	California	Kerwin *et al.* (1994)
An. gambiae	L	*Pythium* sp.	Uganda	Nnakumusana (1985)
"	"	*L. giganteum*	France	Golkar *et al.* (1993)
An. punctipennis	L	"	(North Carolina) USA	Couch & Romney (1973)
An. quadrimaculatus	L	"	(North Carolina) USA	Couch & Romney (1973), Rueda *et al.* (1991)
An. sinensis	L	*Pythium guiyangense*	(Guizhou) China	Su (2008)
An. stephensi	L	*L. giganteum*	USA	Couch & Romney (1973), Rueda *et al.* (1991)
[MYZOCYTIOPSIDALES]				
An. farauti	L	*Crypticola clavulifera*	Australia	Frances (1991)
CHYTRIDIOMYCETES				
[BLASTOCLADIALES]				
An. aconitus	L	*Coelomomyces indicus*	India	Iyengar (1935, 1962)

An. annularis	L, A	*C. anophelesicus*	India	Iyengar (1935)
An. annularis	L	*C. indicus*	"	Iyengar (1935, 1962)
An. barbirostris	L	"	India	Iyengar (1935, 1962)
An. bradleyi	L	*Coelomomyces* sp.	USA	Chapman *et al.* (1969)
An. claviger	L	*C. raffaelei*	Italy	Coluzzi & Rioux (1962)
An. crucians	L	*C. bisymmetricus*	USA	Couch & Dodge (1947)
"	"	*C. cribrosus*	"	"
"	"	*C. dodgei*	"	Couch & Dodge (1947), Chapman & Glenn (1972)
"	"	*C. keilini*	"	"
"	"	*C. lativittatus*	"	"
"	"	*C. punctatus*	USA	Couch & Dodge (1947), Pillai & Rakai (1970), Chapman & Glenn (1972)
"	"	*C. sculptosporus*	"	Couch & Dodge (1947)
An. culicifacies	L	*C. indicus*	Kenya	Service (1977a),
An. earlei	L	*C. lativittatus*	USA	Laird (1961)
An. (Cellia) farauti	L	*Coelomomyces* sp.	Solomon Islands	Pers. comm. from B. Taylor to Maffi & Nolan (1977)
An.	"	*C. cairnsensis*	Australia	Laird (1956a)

farauti				
An. (Cel) farauti	"	*C. indicus*	Australia	Laird (1956a)
An. freeborni	"	*C. dodgei*	USA	Federici (1977)
"	L	*C. punctatus*	"	"
An. funestus	L, A	*C. africanus*	Kenya	Haddow (1942)
"	L	*C. indicus*	Africa	Muspratt (1946 a, b)
An. gambiae	L	*Coelomomyces* sp.	Kenya	Service (1975), (1977a)
"	L, A	*C. ascariformis*	Africa	van Thiel (1954), Rodhain & Gayral (1971)
"		*C. africanus*	Kenya	Haddow (1942),
"	L	*C. grassei*	Africa	Rioux & Pech (1960)
"	L	*C. indicus*	Kenya	Muspratt (1963), Service (1977a),
"	A	*C. walkeri*	Africa	Walker (1938)
An. hyrcanus var. *nigerrimus*	L	*Coelomomyces indicus*	India	Iyengar (1935)
An. (Cel) kolliensis	L	*Coelomomyces* sp.	Solomon Islands	Pers. comm. from B. Taylor to Maffi & Nolan (1977)
An. maculipennis	L	*C. irani*	Iran	Weiser *et al.* (1991)
An. nigerrimus	L	*C. africanus*	Malaysia	Chapman *et al.* (1987)
An. punctipennis	L	*C. cribrosus*	USA	Couch & Dodge (1947)

"	L	*C. quadrangulatus* var. *irregularis*	"	"
An. (Cel) punctulatus	L	*Coelomomyces lairdi* n. sp.	Indonesia (New Guinea)	Maffi & Nolan (1977)
An. punctulatus	L	*Coelomomyces solomonis*	Solomon islands	Laird (1956a, 1961)
An. quadrimaculatus	L	*C. dodgei*	USA (Calif.)	Federici (1977), Federici & Chapman (1977)
"	L	*C. punctatus*	USA (Calif.)	Couch & Dodge (1947), Pillai & Rakai (1970), Federici (1977)
An. ramsayi	L	*C. indicus*	India	Iyengar (1935, 1962)
An. rivulorum	L	*C. indicus*	Africa (Rhodesia)	Muspratt (1946 a, b)
An. rufipes	L	*C. indicus*	"	"
An. sinensis	L	*C. africanus*	Africa USA Malaysia	Rodhain & Gayral (1971), Chapman *et al.* (1987)
An. sinensis	L	*C. raffaelei* var. *parvus*	Japan	Mogi *et al.* (1976)
An. squamosus	L	*C. indicus*	Africa	Muspratt (1946 a, b)
An. squamosus	L	*Coelomomyces numularius*	Angola Africa	Ribeiro & Da Cunha Ramos (2000)

An. stephensi	L	*C. indicus*	Asia	Whisler *et al.* (1999)
An. subpictus	L	*C. anophelesicus*	India (Pondi -cherry)	Iyengar (1962), Vector Control Res. Centre (VCRC) (1978)
An. subpictus	L	*C. indicus*	India, Cambodia	Iyengar (1935, 1962), VCRC (1978), Laird (1959b)
An. tesselatus	A	*C. walkeri*	Java	van Thiel (1954)
An. vagus	L	*C. anophelesicus*	India (Pondi cherry)	Iyengar (1962), (VCRC (1978)
"	L	*C. indicus*	USA India, Phillipines, Thailand	Couch & Umphlett (1963), VCRC (1978), Whisler *et al.* (1999)
An. varuna	L	*Coelomomyces indicus*	India (Bengal)	Iyengar (1935,1962)
An. walkeri	L	*C. quadrangulatus*	USA (Minnesota)	Laird (1961)
"	L	*C. sculptosporus*	"	"
ZYGOMYCETES				
[ENTOMOPHTHORALES]				
An. hispaniola	L	*Entomophthora culicis*	Spain	Lopez-Neyra & Guardiola Mira (1938)
An. maculipennis	A	"	Spain	"
An. stephensi	A	"	USA (New	Kramer (1982)

			York)	
[MUCORALES]				
Anopheles sp.	L	*Mucor stolonifera*	USSR	Bacinskij (1926)
TRICHOMYCETES				
[HARPELLALES]				
An. atroparvus atroparvus	L	*Smittium culicis*	France	Manier (1969)
An. claviger	L	*S. culicis*	"	Manier (1969)
An. gambiae	L	*S. inopinata*	Italy	Colluzzi (1966
An. hilli	L	*S. morbosum*	Australia,	Sweeney (1981)
An. plumbeus	L	*S. culicis*	France	Tuzet *et al.* (1961), Manier (1969)
ANAMORPHIC FUNGI				
Anopheles sp.	L	*Oidium lactis*	USSR	Bacinskij (1926)
An. albimanus	L, A	*B. bassiana*	USA	Clark *et al.* (1967, 1968)
An. albimanus	L	*M. anisopliae*	USA	Roberts (1970, 1974), Ramoska *et al.* (1981)
An. amictus hilli	L	*Culicinomyces* sp.	Australia	Sweeney (1975)
An. amictus hilli	L	*Culicinomyces clavisporous*	"	Cooper & Sweeney (1982), Sweeney (1983)
An. annulipes	L	*Culicinomyces* sp.	"	"
An. aquasalis	L	*Penicillium corylophilum*	Brazil	da Costa *et al.* (1998)

An. aquasalis	L	*Penicillium janthinellum*	Brazil	da Costa *et al.* (1998)
An. arabiensis	A	*M. anisopliae*	Tanzania	Mnyone *et al.* (2009)
An. argyritarsis	L	*Penicillium sclerotirum*	"	Pereira *et al.* (2009)
An. darlingi	L	*Gliocladium viride*	"	Pereira *et al.* (2009)
"	"	*Penicillium citrinum*	"	"
An. farauti	L	*Culicinomyces clavosporous*	Australia	Sweeney (1978)
An. gambiae	L	*Aspergillus clavatus*	Senegal	Seye *et al.* (2009)
An. gambiae	L	*A. parasiticus*	Uganda	Nnakumusana (1985)
"	A	*B. bassiana*	Cameroon	Scholte *et al.*(2003a), Achonduh & Tondje (2008)
"	A	*M. anisopliae*	Kenya, Tanzania	Scholte *et al.*(2003b), Mnyone *et al.* (2009)
An. maculipennis	L	*A. versicolor*	USSR	Kalvish & Kukharchuk (1974)
"	"	*Tolypocladium niveum*	Czeckoslovakia	Matha *et al.* (1988)
"	L	*A. niger*	"	"
"	L	*Geotrichum candidum*	USSR	Kalvish & Kukharchuk (1974).
An. punctipennis	L	*C. clavosporous*	USA	Couch *et al.* (1974)

"	L	*M. anisopliae*	USA	Roberts (1970, 1974)
An. quadrimaculatus	L	*B. bassiana*	USA	Charles (1939)
An. stephensi		*B. tenella*	USA	Pinnock *et al.* (1973)
An. stephensi	L	*Chrysosporium tropicum*	India	Priyanka *et al.* (2001)
"	"	*C. clavosporous*	USA	Couch *et al.* (1974)
"	"	*Fusarium pallidoroseum*	India	Ravindranath & Kapadnis (1991)
"	"	*F. semitectum*	India	Sur *et al.* (1999)
"	"	*Geotrichum candidum*	India	"
"	"	*L. giganteum*	USA	Couch and Romney (1973)
"	L, A	*M. anisopliae*	India	Roberts (1970, 1974), Daoust & Roberts (1982), Kannan *et al.* 2008
"	L	*Trichophyton ajelloi*	India	Mohanty & Prakash (2000)
An. subpictus	A	*A. parasiticus*	India	Hati & Ghosh (1965)

(E = egg; L = larva; P = pupa; A = Adult)

Table 5: List of few mosquito species of Subfamily *Culicinae* on which fungal species were detected or tested either in laboratory or field.

Mosquito Host	Host sta-ge infec-ted	Mosquito-pathogenic fungi [Order] *Species*	Locality	References
OOMYCETES				
[SAPROLEGNIALES]				
Ae. aegypti	L	*Leptolegnia chapmanii*	USA (South Carolina), Argentina	McInnis & Zattau (1982), López Lastra *et al.* (2004)
Ae. albopictus	L	*Leptolegnia* sp.	Florida	Fukuda *et al.* (1997)
Ae. berlandi	L	*Saprolegnia declina*	France	Rioux & Achard (1956)
Ae. cantans	L, P	*Saprolegnia thuretii*	USSR	Kalvish & Kukharchuk (1974)
Ae. cinereus	L, P	*Saprolegnia monoica*	"	"
Ae. detritus	L	*Saprolegnia declina*	France	Rioux & Achard (1956)
Ae. excrucians	"	*Saprolegnia monoica*	USSR	Kalvish & Kukharchuk (1974)
"	L, P	*Saprolegnia hypogyna*	"	"
Ae. geniculatus	L	*Saprolegnia* sp.	Britain	Marshall (1938)

Ae. geniculatus	L	*Saprolegnia declina*	France	Rioux & Achard (1956)
Ae. rusticus	L	*Saprolegnia* sp.	Britain	Marshall (1938)
Ae. triseriatus	L	*Leptolegnia chapmanii*	USA	Fukuda *et al.* (1997)
Ae. vexans	"	*Saprolegnia ferax*	Canada	Goettel (1987a)
Ochlerotatus albifasciatus	"	*Leptolegnia chapmanii*	Argentina	López Lastra *et al.* (2004)
Oc. crinifer	"	"	"	"
Oc. taeniorhynchus	"	"	Louisiana, Florida	Seymour (1984), Lord *et al.* (1988)
Oc. triseriatus	"	"	India	Bisht *et al.* (1996)
Psorophora cyanescens	L	"	Argentina	López Lastra *et al.* (2004)
Ps. ferox	"	*Leptolegnia chapmanii*	"	"
Culex apicinus	L	*Leptolegnia chapmanii*	Argentina	López Lastra *et al.* (2004)
Cx. castroi	"	"	"	"
Cx. dolosus	"	"	"	"
Cx. fastigans		"	Uganda	Nnakumusana (1986)
Cx. modestus	L	*Saprolegnia anispora*	USSR	Kalvish & Kukharchuk (1974)
"	"	*Saprolegnia thuretii*	"	"

Cx. pipiens	"	"	"	"
"	"	*Leptolegnia chapmanii*	Argentina	López Lastra *et al.* (2004)
Cx. pipiens fatigans	"	*Saprolegnia monoica*	Australia	Hamlyn-Harris (1932)
Cx. pipiens pipiens	L, P, A	*Saprolegnia thuretti*	USSR	Kalvish & Kukharchuk (1974)
Cx. quinquefasciatus	L	*Leptolegnia chapmanii*	USA	McInnis & Zattau (1982), Lord & Fukuda (1990)
Cx. salinarius		"	USA	McInnis *et al.* (1985)
Cs. alaskaensis	L	*Saprolegnia ferax*	Canada	Goettel (1987a)
Cs. alaskaensis	L	*Saprolegnia hypogyna*	"	"
Culiseta annulata	L	*Saprolegnia* sp.	Britain	Marshall (1938)
Cs. morsitans	L	"	Britain	"
M. titillans	"	*Leptolegnia* sp.	"	Lord & Fukuda (1990)
M. dyari	"	*Leptolegnia* sp.	"	McInnis & Zattau (1982), Lord & Fukuda (1990)
Orthopodomyia pulchripalpis	"	*Saprolegnia declina*	France	Rioux & Achard (1956)
[PYTHIALES]				
Ae. aegypti	L	*Pythium* spp.	Uganda	Nnakumusana (1985)

			Taiwan	Chen *et al.* (2004)
"	"	*Pythium guiyangense*	(Guizhou) China	Su (2008)
"	"	*Lagenidium* sp.	Fiji	Goettel *et al.* (1983)
Ae. aegypti	L	*Lagenidium giganteum*	USA, France	Couch & Romney (1973), Umphlett (1973), Washino *et al.* (1976), Rueda *et al.* (1990), Golkar *et al.* (1993)
Ae. africanus	L	*Pythium* sp.	Uganda	Nnakumusana (1985)
Ae. albopictus	L	*Pythium* spp.	Taiwan	Chen *et al.* (2004)
"	"	*L. giganteum*	China	Su (1994)
"	"	*Pythium carolinianum*	(Guizhou) China	Su *et al.* (2001)
"	"	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Ae. atropalpus epactius	"	*L. giganteum*	USA	Couch & Romney (1973)
Ae. elsiae	"	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Ae. formosensis	"	"	"	"
Ae. mediovittatus	L	"	USA	McCray *et al.* (1973), Umphlett (1973)
Ae. melanimon	"	*L. giganteum*	USA	Washino *et al.* (1976)

Ae. nigromaculis	"	"	USA	McCray *et al.* (1973), Christensen *et al.* (1977)
Ae. novoniveus	"	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Ae. polynesiensis	L	*Lagenidium* sp.	Fiji	Couch & Romney (1973), Goettel *et al.* (1983)
Ae. pseudoscutellaris	"	"	"	Goettel *et al.* (1983)
Ae. simpsoni	L	*Pythium* sp.	Uganda	Nnakumusana (1985)
Ae. solicitans	L	*L. giganteum*	USA	McCray *et al.* (1973), Umphlett (1973)
Ae. taeniorhynchus	L	*L. giganteum*	USA	McCray *et al.* (1973), Merriam & Axtell (1982)
Ae. triseriatus	L	"	USA	Couch & Romney (1973), McCray *et al.* (1973), Umphlett (1973)
Armigeres dentatus	E	*Lagenidium* sp.	Malaysia	Mattingly (1972)
Oc. sierrensis	L	*Pythium flevoense*	California	Saunders *et al.* (1988)
Oc. sierrensis	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
Oc. triseriatus	L	"	"	"

Psorophora sp.	L	*L. giganteum*	USA, India	Umphlett & Huang (1972), Umphlett (1973), Couch & Romney (1973)
Cx. mimulus	L	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Cx. minor	"	"	"	"
Cx. nigripalpus		*L. giganteum*	South Carolina	McCray *et al.* (1973), Umphlett (1973))
Cx. pipiens	L	*L. giganteum*	France	Golkar *et al.* (1993)
Cx. pipiens fatigans		"	North Carolina, South Carolina	Couch & Romney (1973), McCray *et al.* (1973), Umphlett (1973)
Cx. pipiens pallens	L	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Cx. pipiens quinquefasciatus	"	"	"	"
"	"	*L. giganteum*	USA	Domnas *et al.* 1974
Cx. pseudovishnui	L	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Cx. quinquefasciatus	L	*Lagenidium* sp.	Fiji	Goettel *et al.* (1983),
"	L	*L. giganteum*	USA, India	Washino *et al.* (1976), Patel *et al.* (1990),

			Africa, UK, China	Rueda *et al.* (1990), Orduz & Axtell (1991), Su (1994)
"	L	*Pythium* sp.	Uganda	Nnakumusana (1985)
"	L	*Pythium carolinianum*	(Guizhou) China	Su *et al.* (2001)
Cx. restuans	L	*L. giganteum*	USA	Couch & Romney (1973), Umphlett & Huang (1972)
Cx. tarsalis	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
Cx. tarsalis	L	*L. giganteum*	USA	Couch & Romney (1973), McCray *et al.* (1973), Umphlett (1973), Woodring & Kaya (1992)
Cx. territans	L	*L. giganteum*	USA	Glenn & Chapman (1978)
Cx. theileri	L	*Pythium guiyangense*	(Guizhou) China	Su (2008)
Cx. tianpingensis	"	"	"	"
Cx. tigripes	L	*Pythium* sp.	Uganda	Nnakumusana (1985)
Cx. tritaenio-	L	*Pythium*	(Guizhou)	Su (2008)

rhynchus		*guiyangense*	China	
Cs. incidens	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
M. dyari	L	*L. giganteum*	Florida	Cuda *et al.* (1997)
Or. californica	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
Uranotaenia anhydor	L	*Pythium sierrensis*	USA	Clark *et al.* (1966)
[MYZOCYTIOPSIDALES]				
Ae. aegypti	L	*Crypticola clavulifera*	Australia	Frances *et al.* (1989), Frances (1991)
Ae. notoscriptus	L	*Crypticola clavulifera*	Australia	Frances (1991)
Cx. annulirostris	L	*Crypticola clavulifera*	Australia	Frances (1991)
Cx. quinquefasciatus	L	*Crypticola clavulifera*	Australia	Frances (1991)
CHYTRIDIOMYCETES				
[BLASTOCLADIALES]				
Aedeomyia catastica	L	*Coelomomyces indicus*	Australia	Laird (1956a)
Ae. aegypti	L	*C. dentialatus*	Srilanka	Couch & Bland (1985)
"	L	*C. indiana*	India	Vector Control Res. Centre (1978)

Ae. aegypti	L	*C. indicus*	Uganda	Nnakumusana (1985)
"	L	*C. stegomyiae*	Singapore, Ceylon Philippines	Laird (1959a), Rajapaksa (1964), Villacarlos & Gabriel (1974), Galloway *et al.* 2001
"	L	*C. stegomyiae* var. *chapmani*	Taiwan	Lien & Lin (1990)
"	L, A	*C. stegomyiae* var. *stegomyiae*	Singapore Ceylon, Canada	Shoulkamy *et al.* (1997), Lucarotti & Shoulkamy (2000)
Ae. (Finlaya) albilabris	L	*Coelomomyces* sp.	Solomon Islands	Pers.commun. from B. Taylor to Maffi & Nolan (1977)
Ae. albopictus	L	*C. quadrangulatus* var. *lamborni*	Malaya	Couch & Dodge (1947)
"	"	*C. stegomyiae*	Singapore, Ceylon	Keilin (1921), Rajapaksa (1964)
"	"	*C. stegomyiae var. chapmani*	Taiwan China	Lien & Lin (1990)
Ae. albopictus	L, A	*C. stegomyiae var.*	Japan China	Laird *et al.* (1992), Ramos *et al.* (1996)

		stegomyiae		
Ae. australis	L	*C. opifexi*	New Zealand	Pillai *et al.* (1976)
"	"	*C. tasmaniensis*	Tasmania	Laird (1956b, 1959b)
Ae. cantans	A	*Coelomomyces* sp.	Southern England	Service (1974), Service (1977b)
Ae. cantans	L	*C. psorophorae*	Southern England	Service (1977b)
Ae. cinereus	L	*C. psorophorae* var. *psorophorae*	Sweden	Popelkova (1982)
Ae. detritus	A	*C. psorophorae*	Southern France	Guilvard *et al.* (1977)
Ae. fitchii		*C. borealis* var. *giganteus*	Canada	Couch & Bland (1985)
Ae. hebrideus	L	*C. finlayae*	New Hebrides	Rodhain & Fauran (1975)
Ae. multifolium	L	*C. stegomyiae* var. *stegomyiae*	New Guinea	Briggs (1968)
Ae. multiformis	L, A	*C. stegomyiae* var. *stegomyiae*	Solomon Islands	Huang (1968)
"	"	*C. polynesiensis*		Pillai & Rakai (1970)
Ae. polynesiensis	L, A	*C. stegomyiae* var. *stegomyiae*	Tokelau Phillipines	Laird (1959b,1960, 1967), Padua *et al.* (1986)

Ae. quadrispinatus	L	"	New Guinea	Briggs (1968)
Ae. rotumae	L	*C. stegomyiae* var. *rotumae*	Rotuma island	Laird (1959b)
Ae. scutellaris	L	*C. stegomyiae* var. *stegomyiae*	Solomon islands	Laird (1956a,1967), Padua *et al.* (1986),
Ae. scatophagoides	L	*C. psorophorae*	Northern Rhodesia	Muspratt (1946 a)
Ae. (Stegomyia) simpsoni	A	*Coelomomyces* sp.	Uganda	McCrae (1972)
Ae. sticticus	A	*C. psorophorae*	Canada	Taylor *et al.*(1980)
Ae. stimulans		*C. borealis* var. *giganteus*	Canada	Couch & Bland (1985)
Ae. taeniorhynchus	L, A	*C. psorophorae*	USA	Lum (1963)
Ae. trivittatus	A, L, P	"	(Manitoba) Canada	Taylor *et al.*(1980)
Ae. variabilis	L	*C. stegomyiae* var. *stegomyiae*	New Guinea	Briggs (1968)
Ae. vexans	L	*C. psorophorae*	USA, USSR, Czechoslo-vakia,	Couch & Dodge (1947), Laird (1961), Weiser & Vavra (1964) Goldberg *et al.* (1975), Mitchell (1976)
Ae. vexans	L	*C. psorophorae*	(Alberta)	Shemanchuk (1959),

		var. *psorophorae*	Canada	Zebold *et al.* (1979)
Armigeres obturbans		*C. stegomyiae*	Singapore	Laird (1959b)
Oc. australis	L	*C. psorophorae* var. *tasmaniensis*	New Zealand	Buchanan & Pillai (1990)
Oc. taeniorhynchus	L	*C. psorophorae* var. *psorophorae*	USA	Roberts (1974), Federici & Roberts (1975),
Opifex fuscus	L	*Coelomomyces opifexi*	New Zealand	Pillai & Smith (1968)
Op. fuscus	L	*C. psorophorae* var. *tasmaniensis*	New Zealand	Buchanan & Pillai (1990)
Ps. ciliata	L	*C. psorophorae*	USA	Couch (1945), Laird (1961)
Ps. howardii	L	*C. psorophorae*	USA	Couch & Umphlett (1963), Lum (1960)
"	L	*C. psorophorae* var. *tasmaniensis*	New Zealand	Buchanan & Pillai (1990)
Cx. dolosus	L	*C. iliensis* var. *indus*	Argentina	Lopez Lastra & Garcia (1997)
Cx. erraticus	"	*C. pentangulatus*	USA	Couch & Umphlett (1963), Ribeiro & Da Cunha Ramos (2000)
Cx. fraudatrix	"	*C. cribrosus*	North Borneo	Laird (1959)

Cx. guiarti	"	*C. angolensis*	Africa	Ribeiro (1992)
Cx. modestus	"	*Coelomomyces* sp.	USSR	Dubitskii *et al.* (1972), Deshevykh (1973)
"	"	*C. iliensis*	USSR	Deshevykh & Dzerzhinskii (1975), Dzerzhinskii *et al.* 1975)
Cx. peccator	"	*Coelomomyces* sp.	USA	Chapman *et al.*(1969)
Cx. pipiens pipiens	A	*Coelomycidium* sp.	USSR	Shcherban & Goldberg (1971), Kupriyanova & Aksenova (1973)
Cx. pipiens	L	*Coelomomyces* sp.	Japan Nagasaki	Mogi & Ega (1974)
Cx. pipiens molestus	"	*C. iliensis*	USSR	Dzerzhinskii *et al.*(1976)
"	"	*C. dubitskii*	"	Shcherbak *et al.* (1977)
Cx. pipiens pipiens	"	*C. iliensis*	"	"
"	"	*C. dubitskii*	"	"
Cx. simpsonii	"	*C. indicus*	Africa	Muspratt (1946 a, b)
Cx. territans	"	*Coelomomyces* sp.	USA	Anderson (1968)
Cx. tritaenio-rhynchus	L	*Coelomomyces* sp.	India (Pondi cherry)	Vector Control Res. Centre (1978)

Cx. tritaenio-rhynchus summorosus		*C. cribrosus*	North Borneo, Singapore	Laird (1959 a, b)
"	L	*C. omorii*	Japan Nagasaki	Mogi *et al.* (1976)
"		*C. quadrangulatus* var. *parvus*	Singapore	Laird (1959a,b)
Cx. univittatus	L	*Coelomomyces* sp.	Kenya	Service (1975)
Culiseta inornata	L, A	*C. psorophorae*	USA Canada	Couch & Dodge (1947)
Cs. inornata	L	*C. psorophorae* var. *psorophorae*	Canada	Shemanchuk (1959), Federici & Roberts (1975)
Cs. inornata		*C. psorophorae* var. *tasmaniensis*		Roberts (1974), Chapman (1985)
Cs. melanura	L	*Coelomomyces* sp.	USA (New York)	Mehter (1978) Pers. comm.
Cs. morsitans		*C. psorophorae*	USA	Laird (1961)
Toxorynchytes rutilus septentrionalis	L	*C. macleayae*	USA	Nolan *et al.*1973
U. barnesi	L	*C. solomonis*	British	Laird (1956a), Maffi &

			Solomon islands	Genga
U. sappharina	L	*C. uranotaeniae*	USA	Couch (1945)
ZYGOMYCETES				
[ENTOMOPHTHORALES]				
Ae. taeniorhynchus	L,P, A	*Entomophthora coronatus*	USA	Lowe & Kennel (1972)
Aedes sp.	L	*Entomophthora culicis*	USSR	Kalvish & Kukharchuk (1974)
Ae. aegypti	A	*Entomophthora culicis*	USA	Kramer (1982)
"	A	*Entomophthora musca*	USA	Steinkraus & Kramer (1987)
Ae. aegypti	A	*Erynia conica*	USA	Cuebas-Incle (1992)
Ae. aegypti	A	*Zoophthora radicans*	France	Dumas & Papierok (1989)
Ae. canadensis	L, P	*Entomophthora aquatica*	USA	Anderson & Ringo (1969)
Ae. communis	A, L	*Entomophthora conglomerata*	USSR France	Sorokin (1877), Lakon (1919)
Ae. detritus	A	*Entomophthora culicis*	Britain	Marshall (1938)
Oc. taeniorhynchus	A	*Entomophthora coronata*	USA	Lowe & Kennel (1972)
Cx. pipiens	A	*Conidiobolus*	Nether-	Mietkiewski & Van der

		destruens	lands	Geest (1985)
Cx. pipiens fatigans (*Cx. pipiens quinquefasciatus*	A,P	*Entomophthora coronatus*	USA (Florida)	Lowe *et al.* (1968), Lowe & Kennel (1972)
Culex sp.	A, L	*Entomophthora conglomerata*	USA	Thaxter (1888)
Culex sp.	A, L	*Entomophthora culicis*	Poland, USA,	Nowakowski (1883), Thaxter (1888), Christophers (1952)
Cx. pipiens	A	*Entomophthora conglomerata*	USSR	Lakon (1919), Brumpt (1941), Il'chenko (1968), Goldberg (1969)
"	"	*Entomophthora destruens*	Czeckoslo - vakia, England France, USA	Weiser & Novak (1964), Novak (1967), Service (1969), Goldberg (1973), Cuebas-Incle (1992)
Cx. pipiens	A	*Entomophthora* spp.	Czeckoslo - vakia, Nether- lands	Teernstra-Eeken & Engel (1967), Novak (1977), Mietkiewski & Van der Geest (1985)
Cx. p. pipiens	L, A	*Entomophthora conglomerata*	USSR	Kupriyanova & Aksenova (1973)

Cx. restuans	A	*Erynia conica*	USA	Cuebas-Incle (1992)
Cs. morsitans	L	*Entomophthora aquatica*	USA	Anderson & Ringo (1969)
TRICHOMYCETES				
[HARPELLALES]				
Ae. aegypti	L	*Smittium culicis*	USA (Kansas)	Williams & Lichtwardt (1972)
"	"	*S. culisetae*	"	"
"	"	*S. inopinata*	Italy	Colluzzi (1966)
"	"	*S. simulii*	USA	Williams & Lichtwardt (1972)
Ae. albopictus	L	*S. culisetae*	"	"
Ae. albifasciatus	L	*S. morbosum*	Argentina	García *et al.* (1994)
Ae. berlandi	L	*S. culicis*	France	Tuzet *et al.* (1961), Manier (1969)
Ae. caspius	"	"	"	Manier (1969)
Ae. detritus	"	"	"	Tuzet *et al.* (1961), Manier (1969),
Ae. geniculatus	"	"	France	Manier (1969)
Ae. melanimon	"	"	USA	Clark *et al.* (1963)
Ae. sticticus	"	"	USA	Williams & Lichtwardt (1972)
Ae. triseriatus	L	*S. culisetae*	USA (Kansas)	M. E. Chapman (cited in Williams &

				Lichtwardt, 1972)
"	"	*S. simulii*	"	"
Ae. vexans	"	*Smittium* sp.	Canada	Goettel *et al.* (1987b)
"	"	*S. culisetae*	Kansas USA	Williams & Lichtwardt (1972), Koontz (2006)
Cx. hortensis	"	*S.* (=*Orphella*) *culici*	France	Tuzet & Manier (1947)
Cx. territans	"	*Smittium* sp.	Canada	Goettel *et al.* (1987a)
Cs. alaskaensis	"	*Smittium* sp.	Canada	Goettel *et al.* (1987a)
Cs. inornata	"	*Smittium* sp.	Canada	Goettel *et al.* (1987a)
Cs. impatiens	"	*S. culisetae*	USA	Lichtwardt (1964)
Cs. minnesotae	"	*Smittium* sp.	Canada	Goettel *et al.* (1987a)
Cs. morsitans	"	*Smittium* sp.	Canada	Goettel *et al.* (1987a)
ANAMORPHIC FUNGI				
Aedeomyia squamipennis	L	*Penicillium citrinum*	Brazil	Pereira *et al.* (2009)
Ae. aegypti	L	*Aspergillus clavatus*	Senegal	Seye *et al.* (2009)
Ae. aegypti	L	*A. parasiticus*	Uganda	Nnakumusana (1985)
Ae. aegypti	A, L, E	*Beauveria bassiana*	Brazil, India	Clark *et al.*(1967, 1968), Sandhu *et al.* (1993), Agarwala *et al.* (1998), Luz *et al.* (2007)

Ae. aegypti	E	*Evlachovaea kintrischica*	Brazil	Luz *et al.* (2007)
"	E	*Isaria farinosa*	Brazil	Luz *et al.* (2007)
"	E	*I. fumosorosea*	Brazil	Luz *et al.* (2007)
"	L, E	*Metarhizium anisopliae*	Brazil, India	Ramoska *et al.* (1981), Daoust *et al.* (1982), Sandhu *et al.* (1993), Silva *et al.* 2004, Luz *et al.* (2007), Santos *et al.* (2009)
"	E	*Paecilomyces carneus*	Brazil	Luz *et al.* (2007)
"	L, E	*Paecilomyces lilacinus*	Brazil, India	Agarwala *et al.*(1999), Luz *et al.* (2007)
Ae. aegypti	E	*Paecilomyces marquandii*	Brazil	Luz *et al.* (2007)
"	"	*Penicillium* sp.	Brazil	Luz *et al.* (2007)
"	E, L	*Penicillium citrinum*	France, " Australia	Riba *et al.* (1984), Mvoutoulou (1992), Russell *et al.* (2001)
"	L	*Penicillium corylophilum*	Brazil	da Costa *et al.* (1998)
"	"	*Penicillium janthinellum*	Brazil	da Costa *et al.* (1998)

"	"	*Tolypocladium cylindrosporum*	Canada	Goettel (1988a)
"	"	*Tolypocladium niveum*	Czeckoslovakia	Matha *et al.* (1988)
"	"	*Tolypocladium terricola*	Czeckoslovakia	Matha *et al.* (1992)
"	"	*Tolypocladium tundrense*	Czeckoslovakia	Matha *et al.* (1992)
Ae. africanus	"	*A. parasiticus*	Uganda	Nnakumusana (1985)
Ae. albimanus	A	*B. bassiana*		Clark *et al.* (1968)
Ae. albopictus	L	*T. cylindrosporum*	France	Ravellac *et al.* (1989)
"	"	*M. anisopliae*	"	"
Ae. atropalpus		*M. anisopliae*		Roberts (1970)
Ae. atropalpus epactius	L	*Culicinomyces clavosporus*	USA	Couch *et al.* (1974), Knight (1980)
Ae. australis	L, A	*T. cylindrosporum*	New Zealand	Gardner & Pillai (1987)
Ae. beklemishevi	L	*A. oryzae*	USSR	Kalvish & Kukharchuk (1974)
Ae. cantans	A	*A. versicolor*	"	"
Ae. cantans	"	*Fusarium oxysporum*	USSR	Kalvish & Kukharchuk (1974)
Ae. cantans	"	*F. semitectum*	"	"
Ae. cinereus	L, P	*A. niger*	USSR	Kalvish & Kukharchuk

				(1974)
Ae. detritus	L	*F. oxysporum*	France Italy	Hasan & Vago (1972), Breaud *et al.* (1980)
Ae. dorsalis	"	*B. tenella*	USA	Sanders (1972), Pinnock *et al.* (1973),
Ae. excrucians	"	*B. bassiana*	USSR	Kalvish & Kukharchuk (1974)
"	"	*A. niger*	USSR	"
"	"	*A. kanagawaensis*	"	"
"	L	*Paecilomyces farinosus*	USSR	Kalvish & Kukharchuk (1974), Pinnock *et al.* (1973)
Ae. fluviatilis	"	*A. ochraceus*	Brazil	de Moraes *et al.* (2001)
"	"	*A. sulphureus*	"	"
"	"	***A. sydowii***	"	Pereira *et al.* (2009)
"	"	*P. citrinum*	"	Pereira *et al.* (2009)
"	"	*Penicillium corylophilum*	"	da Costa *et al.* (1998)
"	"	*Penicillium janthinellum*	"	"
Ae. hexodontus	"	*B. tenella*	USA	Pinnock *et al.* (1973)
Ae. kochi	"	*Culicinomyces bisporalis*	Australia	Sigler *et al.* (1987)
Ae. nigromaculis	A	*B. bassiana*	USA	Clark *et al.* (1967,1968)

Ae. pionyis	L	*Geotrichum candidum*	USSR	Kalvish & Kukharchuk (1974).
Ae. polynesiensis	L	*T. cylindrosporum*	Fiji	Gardner *et al.* (1986)
Ae. punctor	L	*A. niger*	USSR	Kalvish & Kukharchuk (1974)
Ae. rubrithorax	"	*C. clavisporus*	New S.Wales	Frances (1986)
Ae. rupestris	"	*C. clavisporus*	USA, Australia	Goettel *et al.* (1984), Sweeney & Panter (1977)
Ae. sierriensis	A	*B. bassiana*	USA	Clark *et al.*(1967, 1968)
Ae. sierriensis	L	*B. tenella*	USA	Pinnock *et al.* (1973), Sanders (1972)
"	L, A	*T. cylindrosporum*	France	Soarés Jr. (1982)
Ae. simpsoni	L	*A. parasiticus*	Uganda	Nnakumusana (1985)
Ae. sollicitans	"	*M. anisopliae*	USA	Roberts (1970)
Ae. subalbirostris	"	*T. cylindrosporum*	New Zealand	Gardner & Pillai (1987)
Ae. taeniorhynchus	"	*M. anisopliae*	USA	Roberts (1970)
Ae. triseriatus	L	*T. cylindrosporum*	Canada	Nadeau & Boisvert (1994)
Ae. triseriatus	L	*Verticillium lecanii*	USA	Ballard & Knapp (1984)

Ae. vexans	A	*A. versicolor*	USSR	Kalvish & Kukharchuk (1974)
"	L	*B. tenella*	"	"
"	"	*Scopulariopsis brevicaulis*	"	"
"	"	*T. cylindrosporum*	Canada	Goettel (1987b)
Oc. australis	L	"	New Zealand	Gardner & Pillai (1987)
Oc. sierrensis	A	*B. bassiana*	USA	Clark *et al.* (1968)
"	L	*B. tenella*	USA	Pinnock *et al.* (1973)
"	A, L	*T. cylindrosporum*	USA	Soarés (1982),
Oc. triseriatus	L	*T. cylindrosporum*	Canada	Nadeau & Boisvert (1994)
Oc. nigromaculis	A	*B. bassiana*	USA	Clark *et al.* (1968)
"	"	*Oidium lactis*	USSR	Bacinskij (1926)
Ps. confinnis	L	*C. clavosporus*	USA (N. Carolina)	Couch *et al.* (1974)
Culex sp.	L	*Aspergillus* sp.	USA	Speer (1927)
Cx. apicalis	"	*B. bassiana*	"	Dyl'ko (1971)
Cx. exilis	"	*B. bassiana*	"	"
Cx. fatigans	L	*A. parasiticus*	Uganda	Nnakumusana (1985)
Cx. gelidus	A	*A. parasiticus*	India	Hati & Ghosh (1965)
Cx. modestus	L	*A. niger* v. *teigh*	USSR	Kalvish & Kukharchuk (1974)

"	"	*B. bassiana*	"	Saubenova *et al.* (1973)
"	"	*B. tenella*	"	Kalvish & Kukharchuk (1974)
"	"	*Sporotrichum roseolum*	"	".
Cx. molestus	"	*Tolypocladium niveum*	Czeckoslovakia	Matha *et al.* (1988)
Cx. pipiens	L	*A. niger*	USSR	Kalvish & Kukharchuk (1974)
Cx. pipiens	L	*A. versicolor*	USSR	"
"	L, A	*B. bassiana*	"	Dye (1905), Roubaud & Toumanoff (1930), Clark *et al.*(1967, 1968), Saubenova *et al.* (1973), Kalvish & Kukharchuk (1974),
Cx. pipiens	L	*B. tenella*	USA USSR,	Pinnock *et al.* (1973), Kalvish & Kukharchuk (1974)
"	P	*F. culmorum*	Egypt	Badran & Aly (1995)
"	P	*F. dimerum*	"	*id*
"	"	*M. anisopliae*	USSR	Kalvish & Kukharchuk (1974)
"	"	*Paecilomyces farinosus*	"	"

Cx. pipiens fatigans	A	*A. parasiticus*	India	Hati & Ghosh (1965)
"	A	*Cladosporium* sp.	Singapore	Laird (1959b)
"	L	*Culicinomyces* sp.	Australia	Sweeney (1975)
"	"	*C. clavosporus*	USA	Couch *et al.* (1974)
Cx. pipiens molestus	"	*B. bassiana*	USSR	Vansulin (1974)
Cx. pipiens pipiens	"	*Aspergillus niger* v. *teigh*	"	Kalvish & Kukharchuk (1974)
"	"	*B. bassiana*	"	"
Cx. pipiens pipiens	L	*F. oxysporum*	France, Italy	Hasan & Vago (1972), Breaud *et al.* (1980)
"	"	*M. anisopliae*	USA	Roberts (1977), Al-Aidroos & Roberts (1978)
Cx. quinquefasciatus	L	*Aspergillus clavatus*	Senegal (Africa)	Seye *et al.* (2009)
Cx. quinquefasciatus	L	*A. kanagawaensis*	"	de Moraes *et al.* (2001
Cx. quinquefasciatus	L	*A. ochraceus*	Brazil	de Moraes *et al.* (2001)
"	"	*A. sulphureus*	"	"
"	"	*Chrysosporium lobatum*	India	Mohanty & Prakash (2008)
"	"	*Culicinomyces*	Australia	Sweeney (1975)

		sp.		
Cx. quinquefasciatus	"	*C. clavisporus*	U.S.A, Australia	Couch *et al.* (1974), Cooper & Sweeney (1982, 1986)
"	A	*F. pallidoroseum*	India	Mohanty *et al.* 2008
"	L	*M. anisopliae*	Florida	Ramoska *et al.* (1981); Lacey *et al.* (1988)
"	"	*Penicillium corylophilum*	Brazil	da Costa *et al.* (1998)
"	"	*Penicillium janthinellum*	"	"
Cx. quinquefasciatus	L	*Trichophyton ajelloi*	India	Mohanty & Prakash (2000)
Cx. restuans	L	*C. clavosporus*	USA	Couch *et al.* (1974)
Cx. tarsalis	A, L	*B. bassiana*	USA	Clark *et al.*(1967,1968)
"	L	*B. tenella*	USA	Pinnock *et al.* (1973)
"	L	*T. cylindrosporum*	France	Soarés (1982), Soarés Jr.(2006)
Cx. territans	"	*C. clavosporus*	USA (N. Carolina)	Couch *et al.* (1974)
"	"	*T. cylindrosporum*	Canada	Goettel (1987b)
Cx. tigripes	"	*A. parasiticus*	Uganda	Nnakumusana (1985)
Cx. tritaeniorhyn-chus	"	*B. bassiana*	India	Sandhu *et al.* (1993)

Mansonia titillans	"	*Acremonium* ***kiliense***	Brazil	Pereira *et al.* (2009)
"	"	***Fusarium sacchari* var. *sacchari***	Brazil	Pereira *et al.* (2009)
"	"	***Fusarium merismoides* var. *merismoides***	Brazil	Pereira *et al.* (2009)
"	"	*M. anisopliae*	"	"
Culiseta sp.	L	*B. tenella*	California	Sanders (1972)
Cs. alaskaensis	"	*Cladosporium linicola*	USSR	Kalvish & Kukharchuk (1974)
Cs. incidens	"	*B. tenella*	California	Pinnock *et al.* (1973),
Cs. inconspicua	"	*C. clavisporus*	Australia	Frances (1986)
Cs. inornata	"	*C. clavisporus*	Canada	Goettel *et al.* (1984)
"	"	*M. anisopliae*	USA	Roberts (1970, 1974)
"	"	*T. cylindrosporum*	Canada	Goettel *et al.* (1987b)
Cs. melanura	"	*C. clavosporus*	USA	Couch *et al.* (1974)
Uranotaenia sp.	L	*Penicillium melinii*	Brazil	Pereira *et al.* (2009)
Uranotaenia sapphirina	L	*C. clavosporus*	USA	Couch *et al.* (1974)

(E = egg; L = larva; P = pupa; A = Adult)

Table 6: Genera of fungi found infecting mosquito host.		
S.No.	**Sub-family *Anophelinae***	**Sub-family *Culicinae***
1	*Leptolegnia* spp.	*Leptolegnia chapmanii*
2	*Saprolegnia* sp.	*Saprolegnia* spp.
3	*Aphanomyces laevis*	NR
4	*Pythium* spp.	*Pythium* spp.
5	*Lagenidium giganteum*	*Lagenidium giganteum*
6	*Crypticola clavulifera*	*Crypticola clavulifera*
7	*Coelomomyces* spp.	*Coelomomyces* spp.
8	*Entomophthora culicis*	*Entomophthora* spp.
9	NR	*Erynia conica*
10	NR	*Conidiobolus destruens*
11	*Smittium* spp.	*Smittium* spp.
12	*Mucor stolonifera*	NR
13.	NR	*Acremonium kiliense*
14	*Beauveria* spp.	*Beauveria* spp.
15	*Oidium lactis*	*Oidium lactis*
16	NR	*Evlachovaea kintrischica*
17	NR	*Isaria* spp.
18	*Culicinomyces clavosporus*	*Culicinomyces clavosporus*
19	*Gliocladium viride*	NR
20	*Metarhizium anisopliae*	*Metarhizium anisopliae*
21	*Penicillium* spp.	*Penicillium* spp.
22	NR	*Paecilomyces* spp.

23	*Aspergillus* spp.	*Aspergillus* spp.
24	*Geotrichum candidum*	*Geotrichum candidum*
24	NR	*Tolypocladium cylindrosporum*
25	*Chrysosporium tropicum*	*Chrysosporium lobatum*
26	*Fusarium* spp.	*Fusarium* spp.
28	*Trichophyton ajelloi*	*Trichophyton ajelloi*
29	NR	*Scopulariopsis brevicaulis*
30	NR	*Sporotrichum roseolum*
31	NR	*Cladosporium* sp.
Total	21 genera	28 genera

NR= not reported; sp. = species; spp. = species (plural)

hydrophobic conidia. *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumoroseus* also have hydrophobic conidia (Boucias *et al.*, 1988). The hydrophilic conidia of *Hirsutella thompsonii* after infecting mites produce a network of mycelia extending away from the fungus killed mites on the leaf substrate (McCoy & Kanavel, 1969). The coelomomycete *Aschersonii aleyrodis* produces slimy masses of conidia in pycnidia after infecting sessile whiteflies (Fransen, 1987). Entomphthoralean epf which infect a spectra of insects (King & Humber, 1981), actively discharge primary conidia away from the dead hosts. In aquatic fungi like *Aphanomyces*, *Lagenidium*, *Leptolegnia* and *Coelomomyces* motile spores are infectitious propagules which bind to and encyst on host cuticles. The motile biflagellate zoospores of *L. giganteum* infect all major genera of mosquitoes *Aedes, Anopheles, Culex*, *Culiseta* and *Psorophora*. The biflagellate zygote of *Coelomomyces psorophora* selectively binds and encysts on certain mosquito hosts and the motile haploid zoospore of the same encysts on the alternate copepod host to complete its life cycle (Zebold *et al.*, 1979).

2.4.2 Site of infection and spore attachment:

Generally the conidia of epf attach randomly to the host epicuticle. *Culicinomyces clavosporus* conidia attach over entire foregut cuticular surface of *Culex fatigans* (Sweeney, 1975). Certain areas of the insect cuticle are vulnerable to penetration by propagules of epf (Fig. 2). The selected sites of infection can be the intersegmental membranes (Wraight *et al.*, 1990), areas under the elytra (Butt *et al.*, 1995) and the buccal cavity (Schabel, 1976). In aquatic entomopathogens host recognition seems to be the rule. A tendency of the motile zoospores of *Leptolegnia chapmanii* to aggregate and encyst on the cervical collar and intersegmental regions of mosquito larvae is seen (Zattau & McInnis, 1987; Lord & Fukuda 1988).

Ultimate sites of penetration by the fungus may be affected by insect behaviour. The sites for penetration of *Beauveria brongniartii* on larvae of the cockchafer *Melolontha melolontha* were the mouth and anus (Delmas, 1973). In field collected larvae of the same species, the most frequent sites of infection were the membranes between the head capsule and thorax or on the intersegmental membrane of appendages which are well protected preventing scraping the infectitious propagule off the exposed cuticle due to the larval burrowing habit. In mosquito larval hosts *Tolypocladium cylindrosporum* attaches preferentially to the head region reflecting the feeding behaviour of the host larvae rather than cuticle recognition (Goettel, 1988a). The location where the inoculum positions itself determines the probability of infection and the speed of the kill. Targeting of the inoculum is a vital aspect to consider while establishing bioassay protocols.

2.4.3 Fungal invasion through the host cuticle:

Fungi are unique amongst the entomopathogens as they infect their hosts primarily through the external cuticle. The tough, flexible and waterproof cuticle of the insects is a barrier against the outside world formed by the basal epidermal cells (Neville, 1975).The resistance to fungal entomopathogens by the insect host is determined by the cuticular thickness, the degree of cuticular cross-linking within the cuticular laminae (i.e. cuticular strength) and the degree of sclerotisation in the cuticle (Hajek & St. Leger, 1994).

An outer epicuticle and an inner procuticle are the two layers of the cuticle. A waxy layer containing fatty acids, lipids and sterols covers the epicuticle. Epicuticle a complex thin structure contains phenol-stabilized proteins but lacks chitin (Hackmann, 1984). Majority of the cuticle is formed by the procuticle. Embedded in a protein matrix along with lipids and quinones are chitin fibrils in the procuticle

(Neville, 1984). 70% of the cuticle is proteinaceous in nature. The helical organization of chitin in many areas of the cuticle gives rise to a laminate structure.

The *in vivo* development cycle of commercially important epf, such as *B. bassiana* and *M. anisopliae*, involves sequentially:

1. Adhesion of conidia (spores) to the host cuticle: Generally for entomopathogenic fungi host location is a random event and attachment is a passive process with the aid of wind or water. Spore attachment to the insect cuticle is a two-step process. Non-specific adhesion is followed by specific adhesion. The non-specific adhesion of hyphomycetous conidiospores to insect cuticle was mediated by hydrophobic interaction between the conidial cell wall and epicuticle of the insect (Boucias *et al.*, 1988). Hydrophobic conidia of many pathogens will bind in a non-specific manner to the epicuticle of both susceptible and resistant host insects (Boucias *et al.*, 1988; Boucias & Pendland, 1991). However, production of penetrant germ tubes does not normally occur on non-host organisms. Specific cell adhesion attaches the spore more firmly to the insect surface. Fungal pathogens possess specific proteinaceous receptors called adhesions or lectins which recognize glycoproteins or glycolipids on the surface of target cells (Jenq *et al.*, 1994). In *M. anisopliae* spore isolates specific surface antigens are required for pathogenicity and may play an important role in host specificity (Rath *et al.*, 1995). In the Entomophthorales, the hydrophilic nature of conidia is thought to be mediated by a mucilaginous coat released upon attachment to cuticle surfaces, which acts as a glue mediating attachment (Eilenberg *et al.*, 1986; Latge *et al.*, 1988). It was found that dry spores of *B. bassiana* possess an outer layer composed of interwoven fascicles of hydrophobic rodlets. This rodlet layer is found in the conidial stage only and has not been detected on the vegetative cells.

2. Germination of the conidia: Once attached to the host surface the spore must germinate for the infection to progress. The spore is activated by favourable environmental conditions followed by isotropic growth which is observed as swelling involving water uptake and growth. In *M. anisopliae* swelling in water triggered the release of cuticle degrading enzymes (St. Leger *et al.*, 1991a). The unipolar growth of the emergent germ tube which can be easily seen marks the end of germination. Ambient temperature and relative humidity (RH) influence spore germination. The conidiospore of *B. bassiana* germinates between 25-30° C. Usually high RH is required but there are many reports of germination at low RH leading to infection as in *B. bassiana* on adult migratory grasshopper *M. sanguinipes* (Marcandier & Khachatourians, 1987) and chinch bug *B. leucopterus* (Ramoska, 1984). This may be due to microenvironments of moisture on specific cuticular surface (hair, folds, depressions, etc.) that allows germination to occur at low RH. Specific nutrient requirements like specific carbon source are seen in the germ tube and the peg formation Induction of appressorial formation by low levels of nitrogenous compounds against hard hydrophobic surfaces and suppression of infection related morphogenesis and cuticle penetration at high levels is seen. Long chain fatty acids (C10:0 to C18:1) from cuticular extracts of *M. sanguinipes* were used by *B. bassiana* for growth while short chain fatty acids specifically inhibited spore germination (C4:0 to C9:0) (Smith & Grula, 1982). Further the cuticular hydrocarbons have to be degraded for host cuticle penetration. In general epf strains must have resistance to the epicuticular hydrocarbons and lipids for successful penetration. More specific requirement for germination is seen in epf with restricted host ranges (St. Leger, 1989). The requirement of oleic acid for germination restricts *Erynia variabilis* to small dipterans (Kerwin, 1984).

3. Production of germ tube and appresorium (penetration structure): The length of the germ tube and its differentiation into an appressorium are dependent on the surface topography of the insect and the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) (St. Leger *et al.*, 1991b). Conidia germinate on the host surface differentiating into an infection structure appressorium. To concentrate physical and chemical energy over a very small area appressorium represents an adaptation so that ingress may be achieved efficiently. Hence, in establishing a pathogenic interaction with the host, formation of the appressorium plays an important role. Host surface topography is indicated in the appressorium formation (St. Leger *et al.,* 1991b).

On conductive surfaces (hydrophobic or nutrient poor substances) conidia produce an appressorium (Butt *et al.*, 1995). Depending on the fungal strain and/or species as well as cuticular cues the size and shape of the appressorium varies (Butt *et al.*, 1995). Almost all strains of *M. anisopliae* produce appressoria whereas few strains of *B. bassiana* and *Verticillium lecanii* produce appressoria. Hyphae penetrate the cuticle directly without producing appressoria in *Nomuraea rileyi*. Germ tubes of many entomopathogens produce mucilage which is believed to bind the appresorial cells to the cuticle (Zacharuk, 1970; Travland, 1979; St. Leger *et al.*, 1989). *Coelomomyces psorophorae* formed appressoria in the larvae of *Culiseta inornata* and this was accompanied by the formation of exocellular mucilage (Travland, 1979). Phenoloxidase activity, an immune effector system responsible for producing melanin, has been detected in insect cuticle (Ashida & Brey, 1995); however its exact role is not known. Fungal germ tubes are melanised as they pass through procuticle before they enter the haemocoel due to the activity of this enzyme (Golkar *et al.*, 1993).

4. Penetration of the cuticle:

A combination of mechanical pressure and the action of cuticle degrading enzymes is required for the penetration of intact host cuticle depending on the cuticle encountered (Charnley, 1984). In wireworm cuticle the wax layer disappears beneath the appressoria of *M. anisopliae* indicating enzyme activity (Zacharuk, 1970). Certain Entomophthoralean fungi produce triradiate and tetraradiate fissures in the host cuticle due to physical penetration (Brobyn & Wilding, 1983). Penetrant structures expand laterally in the outer procuticle producing penetrant plates which can cause fractures favouring penetration (Brobyn & Wilding, 1983; Brey *et al.*, 1986). The degree of sclerotization (cross-linking of proteins by quinones) influences penetrability strongly. Insects with heavily sclerotized body segments are invaded via arthroidal membranes or spiracles (Charnley, 1984; St. Leger, 1991). EPFs produce extracellular enzymes which are involved in degrading insect cuticle. Lipases, proteolytic enzymes and chitinases are secreted which have a concerted action along with turgor pressure for penetration. In *B. brongniartii* lipase deficient mutants were not virulent against common cockchafer, *Melolontha melolontha* larvae.

M. anisopliae produces multiple isozymes of both basic chymoelastase and acidic trypsin-like proteases (St. Leger *et al.* 1987b, 1994). In *M. anisopliae* and *B. bassiana* basic proteases have a greater role in cuticle degradation, in the latter being an important virulence factor. The role of chymoelastase of *M. anisopliae* in causing mortality to mosquito larvae was studied by Mohanty *et al.* (2008a). Investigations on *M. anisopliae, V. lecanii* and *B. bassiana* with cuticle of *M. sanguinipes* showed that all proteases required carboxyl group for adherence to cuticle (Bidochka & Khachatourians, 1990). Cuticular tyrosine-hydroxyl groups were required for maximal adsorption of the protease in *M. anisopliae* and *V. lecanii*.

Multiple chitinase isozymes with varied molecular weights were produced by the epf *Aspergillus flavus*, *M. anisopliae* and *B. bassiana* (St. Leger *et al.*, 1993). Specific or combination of isozymes may have

greater significance compared to the total chitinase activity. Pre-treatment of insect cuticle with protease enhanced chitinase-mediated hydrolysis suggesting protein matrix protects chitin from degradation (St. Leger *et al.*, 1986b).

5. Vegetative growth within the host haemocoel: If the fungus is successful in penetrating it continues to increase in biomass at the expense of the host. Alongwith hyphal proliferation it can form blastospores or hyphal bodies to colonize insect coelom (Pendland & Boucias, 1982; Pendland *et al.*, 1994). *In vivo* generated blastospores (distinct but similar to the rich broth-produced blastospores), in the insect hemolymph are able to evade recognition by insect hemocytes and display altered membrane characteristics (Boucias *et al.*, 1988; Hung & Boucias 1992; Hung *et al.*, 1993).

6. Production of externally-borne conidia upon death of the host: Upon death of the host the mycelia will fill the insect cuticle, by then the different tissues will be damaged. Under favourable condition the mycelia break through the cuticle producing aerial conidiospores which may initiate infections in other target insects.

2.4.4 Invasion through the gut and factors influencing germination:

Apart from penetration of epf through body wall of insects penetration through the gut wall also has been reported (reviewed by Dillon & Charnley, 1991). In terms of barriers the insect digestive system is the least well-defended region physically and is constantly exposed to non-self (i.e. food). A relatively thin layer of cuticle lining the foregut and hindgut bestows some degree of protection (Chapman, 1998). The endodermal midgut and its associated structures, are vulnerable as the epithelial surface is not lined with cuticle, often providing parasites with specific entry points into the host (Han *et al.*, 2000). Physical protection in the midgut is afforded by the delicate cuticular peritrophic membrane (pm). The composition of gut cuticle is

similar to that of external cuticle but in the former an outer waxy layer is lacking as it has to be permeable and the phenolic cross-linkages between the protein molecules are absent making the gut cuticle softer (Bignell, 1984).

In case of external cuticle adhesion of spore precedes germination whereas in the gut germination can occur before adhesion. The fungal propagule must be retained in the gut for germination to occur increasing the inoculum potential. Insects with short food transit times show long-term retention of spores (Veen, 1968; Schabel, 1976; Dillon & Charnley, 1986) as a large portion of the inoculum is flushed with the meal before the conidia can adhere or germinate. In *Aedes aegypti* the pm may play an important role in prolonging the retention of spores in the gut.

The insect gut has a varying environment in different regions. The fungal viability within the gut is affected by a number of interacting factors. Conidial fungi generally grow between pH 4 and 9 (Cochrane, 1958) a range found in many insect guts (Bignell, 1984). Oxygen unavailability limits germination of conidia (Hall, 1981). It is postulated in cockroaches that the hindgut which harbours anaerobic microbiota prevents fungal colonization of the hindgut (Dillon & Charnley, 1991).

Nutrient availability influences germination of conidia. Spores in the foregut and midgut may have access to ingested or digested nutrients which are lacking in hindgut. More over they will have to compete with other microbiota in the hindgut for unabsorbed nutrients (Dillon & Charnley, 1991). Iron is important for germination of conidia and iron deficit environment due to host gut microbes results in fungistasis (Griffiths, 1983). Colonization of gut wall by bacteria may prohibit adhesion of fungi (Savage, 1969). High osmotic pressure is unfavourable for conidial germination. In the gut of mycophagous insects (Kukor & Martin, 1987), ants (Martin *et al.*, 1976)

and cockroaches (Waterhouse & McKellar, 1961) chitinase has been located which have important implications for the fate of fungal spores.

In aquatic insects gut infection is more common. *Culicinomyces clavisporous* producing submerged conidiospores exclusively infects dipteran larvae (Culicidae, Ceratopogonidae, Chironomidae) via foregut, hindgut or anal papillae (Sweeney, 1979; Knight, 1980). Mosquito larvae being filter feeders ingest large numbers of conidia rapidly to the degree that the gut is packed with conidia (Agudelo-Silva & Wassink, 1984; Lacey *et al.* 1988). In the gut of *Culex pipiens* about 89% of conidia of *Metarhizium anisopliae* were disrupted or digested (Crisan, 1971). In *Aedes* spp. some strains of *Tolypocladium* cylindrosporum are shown to cause gut infections (Samson & Soares, 1984).

2.5 Enzymes produced by entomopathogenic fungi:

In the past few decades significant progress has been made in understanding enzymes involved in the penetration process. Detailed studies on cuticle degrading enzymes in *Metarhizium anisopliae* have been carried out using locust cuticle as carbon source. Extracellular enzymes appear sequentially in *Metarhizium liquid* culture. Esterase and proteolytic enzymes such as endopeptidase, aminopeptidase and carboxypeptidase were produced within 24 h. *N*-acetylglucosaminidase followed and chitinase and lipases were produced 3-5 days later. The order of cuticle constituents corresponds to the sequence of extracellular production of the cuticle degrading enzymes (St. Leger *et al.*, 1986a). Significant variation was noted in the production profiles of different strains of *B. bassiana (*Gupta *et al.*, 1992). *B. bassiana* mutants producing reduced levels of cuticle degrading enzymes were still pathogenic to their hosts making it difficult to correlate activities with pathogenicity (Heale *et al.*, 1989).

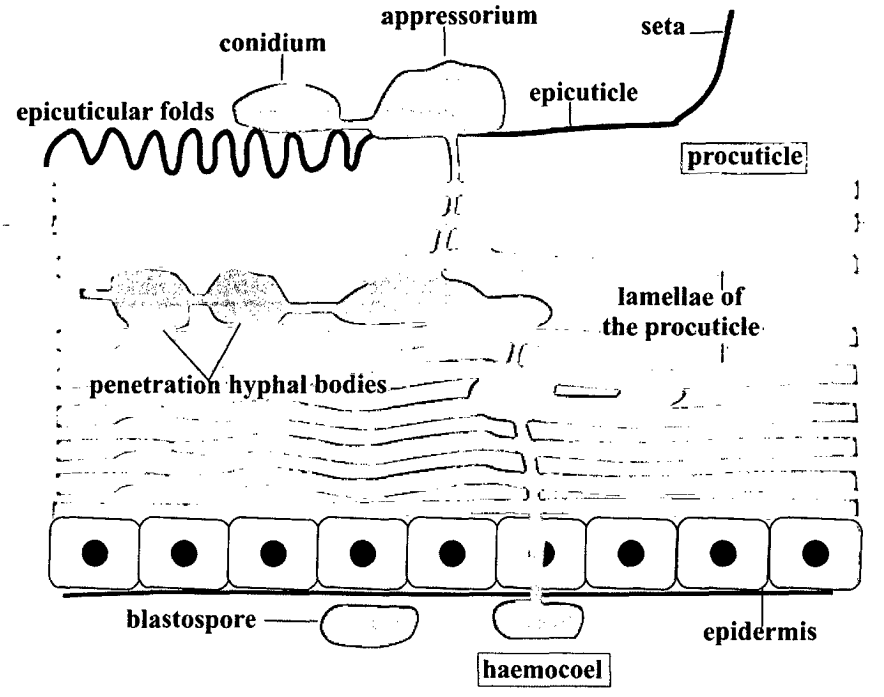


Fig. 1: Schematic diagram of structure of insect cuticle invaded by fungal conidia. Modified from Charnley (2003).

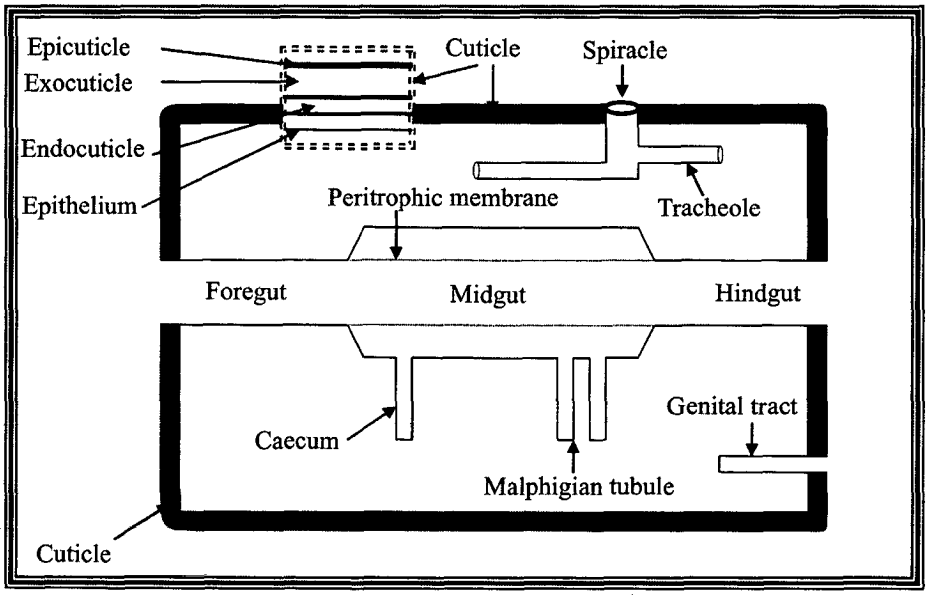


Fig. 2: Schematic diagram of usual sites of fungal infection in mosquito larva. Modified from Siva-Jothy *et al.* (2005).

In *M. anisopliae* Silva *et al.* (1989) did not find any correlation between protease and virulence but found a better correlation between amylase activity and virulence than between lipase activity and virulence. In *V. lecanii* the ability to degrade lipid and protein was not correlated with virulence or avirulence because all isolates produced these enzymes Jackson *et al.* (1985). The role of chitinases is also unclear. In *N. rileyi* El-Sayed *et al.*, (1989) showed that virulent and avirulent isolates had similar chitinolytic activity throughout their growth except at penetration time, virulent isolates consistently had significantly higher levels of activity. In the germlings of virulent strains of *B. bassiana*, *M. anisopliae* and *N. rileyi* Coudron *et al.* (1984) detected low levels of chitinase activity. Studies of protease production in infected cuticles associated with cuticle degradation, the effects of protease inhibitors on pathogen behaviour, the analysis of protease-deficient mutants or transgenic strains over-expressing subtilisin-like protease Pr1 have given an insight into the role of proteases in penetration (St. Leger *et al.*, 1996). Cloning, identification and manipulation of specific protease genes of *M. anisopliae*, particularly those of Pr1 which is produced by many other entomogenous, hyphomycete fungi is the focus of recent studies (Joshi *et al.*, 1995, 1997).

M. anisopliae, *B. bassiana*, *V. lecanii*, *Tolypocladium niveum*, *Paecilomyces farinosus* produce a subtilisin-like protease in nutrient limiting conditions which can be repressed by addition of certain low molecular weight compounds showing that enzyme regulation is at transcriptional level (Charnley, 2003). In the expression of the cuticle degrading enzymes by *M. anisopliae* ambient pH is also a major determinant with each enzyme (Pr1, trypsin-like protease Pr2, metalloproteases, aspartyl proteases, aminopeptidase and chitinases) being synthesized only at the pH at which it functions effectively (St. Leger *et al.*, 1998). Production of ammonia and

organic acids was also seen. The fungal proteases may be limited by various factors in the cuticle including phenoloxidase and protease inhibitors. In hyperproductive Pr1 mutant *M. anisopliae* activation of phenoloxidase cascade with production of large amount of melanin resulted in early larval death with significant reduction in spore production on the cadaver (St. Leger *et al.*, 1996). In *Nomuraea rileyi* a specific enzyme prevents moulting in silkworm *Bombyx mori* larvae keeping the host feeding and in an optimum nutritional state (Kiuchi *et al.*, 2003).

2.6 Fungal Toxins:

The term "toxin" (Gr: "toxikon" = arrow poison) is used to refer specifically to a particular protein produced by some higher plants, fungi, animals and pathogenic bacteria. In bacteria the term describes high molecular weight proteins (Roberts, 1980) whereas in plants and fungi it is restricted to low molecular weight compounds that are bioactive in low concentrations and products of secondary metabolism (Graniti, 1972). In the haemolymph of insects the fungi grow as hypal bodies or blastospores rather than in myceliar form as this aids in dispersal, colonization, nutrient acquisition and to dissuade host immune system. Without penetrating the organs when the host is killed after a limited period of sparse vegetative growth than the toxins may cause death (Roberts, 1980).

After the immunosuppressive agent cyclosporin A was discovered from the filamentous fungus, *Tolypocladium inflatum* in 1980 it turned out to be the decade of resurgence in the discovery of useful compounds from fungal sources. Cyclosporins, cyclic undecapeptides were isolated recently from *Tolypocladium cylindrosporum* a pathogen of mosquito larva. The entomopathogenic fungi *Metarhizium anisopliae* (Roberts, 1966; 1967), *Beauveria* spp. (Hamill *et al.*, 1969), *Tolypocladium* spp.

(Weiser & Matha, 1988a, b; Krasnoff & Gupta, 1991; Jegorov *et al.*, 1993), *Verticillium lecanii* (Claydon & Grove, 1982), *Fusarium* spp. (Peeters, 1989), etc. produce secondary metabolites. There are a number of secondary metabolites like bassianolides (Suzuki *et al.*, 1977), beauvericin (Grove & Pople, 1980; Gupta *et al.*, 1995), destruxins (DTX) (Amiri *et al.*, 1999), enniatins (Visconti *et al.*, 1992), efrapeptins (Krasnoff & Gupta, 1991), fusaric acid (Claydon *et al.*, 1977), Kojic acid (Beard & Walton, 1969; Dowd, 1999) phomalactone (Krasnoff & Gupta, 1994), viridoxins (Gupta *et al.*, 1993), etc. having insecticidal activity. Isolates of *Aspergillus flavus* and *Aspergillus parasiticus* produce aflatoxins which showed delayed development, diminutive pupal and adult size, reduced fecundity and sterility in insects (Wright *et al.*, 1982). Viridoxins produced by *Metarhizium anisopliae* var. *flavoviride* are insecticidal to Colorado Beetle *Leptinotarsa decemlineata*. The mite pathogen *Hirsutella thompsonii* produces Hirsutellin A, a non-glycosylated thermostable protein which is insecticidal to a range of insects including aphids, fruit flies and mosquitoes (Mazet & Vey, 1995). Tolypin from *Tolypocladium niveum* was reported to be larvicidal against mosquitoes and blackflies (Matha *et al.*, 1988). In the *Culex pipiens autogenicus* larvae the depsipeptide beauvericin from *Beauveria bassiana* caused ultrastructural changes in the midgut epithelium and mortality (Zizka & Weiser, 1993). Enniatins produced by *Fusarium lateritium* a scale insect pathogen are less active against the larvae of *Ae. aegypti* than beauvericin, but more insecticidal against the blowfly larvae *Calliphora erthrocephala* (Grove & Pople, 1980). Metabolites of fungi and actinomycetes active against 3rd instar mosquito larvae of three important vectors *Anopheles stephensi* (Liston), *Culex quinquefasciatus* (Say) and *Aedes aegypti* (Linnaeus) have been screened in India by Vijayan & Balraman (1991), Mohanty & Prakash (2004).

Diverse effects of the toxins on various insect tissues were observed. DTX depolarizes the *lepidopteran* muscle membrane by activating calcium channels. In addition, function of insect haemocytes can be inhibited by DTX (Bradfisch, 1990). Hirsutellin is cytolytic to insect cells but does not lyse other prokaryotic or eukaryotic cells tested (Liu *et al.*, 1995). From parasitized insects, presumably there are still many toxins that are to be isolated and except DTXs, their relevance to pathogenicity remains to be established.

2.7 Epizootology of fungal diseases in insects:

In field populations of insects epizootics of fungi can occur, which can be important in the natural regulation of insects. Complex interaction between the host, the pathogen and the environment over time (i.e. the disease tetrad) results in epizootics and for most fungal species not much is known about the factors responsible for the initiation and continuance of the epizootics.

When the pathogen, host and environmental conditions act in concert infection occurs. More than 100 species of fungi are present in the genus Entomophthora, many of which cause epizootics in grasshoppers, mosquitoes, aphids and houseflies. *Nomuraea rileyi* is the best studied entomopathogenic fungus from an ecological perspective (Carruthers & Soper, 1987). The timing of initial infection of the host population, the developmental lag associated with the disease incubation period, which is related to the host instar, the rate of spread of the pathogen and various environmental factors are all known to control the occurrence of epizootics (Ignoffo *et al.*, 1977; Kish & Allen, 1978; Ignoffo, 1981). Kish & Allen (1978) developed one of the first quantitative epizootiological models based on a series of stimulus-response experiments.

2.8 Factors affecting infectivity:

The Fungal Pathogen

Pathogenicity is the qualitative ability of a pathogen to cause disease. Host-pathogen compatibility is a major determinant of fungal pathogenicity. Virulent strains must be compatible with the target host to cause infection. The ability of the pathogen to recognise its host and to overcome the defense barriers influences compatibility. The impact of environmental factors on host- pathogen interactions affects the disease causing ability of a pathogen. The host spectra varies according to the fungal species *N. rileyi* almost exclusively infects lepidopterans whereas *B. bassiana* and *M. anisopliae* have much wider host ranges which is now attributed to the diverse assemblage of genotypes found in these.

Dose– related mortality

To overcome the host it is presumed that there exists a threshold whereby a certain number of propagules are necessary. Susceptibility of most insects is dependent on spore dosage. In the field, the spore density must be sufficiently high to ensure that an insect will contact sufficient infective propagules to exceed the threshold to cause disease. Two important considerations in strain selection are virulence and ecological fitness. Less inoculum is required by virulent strains to cause infection (Low LD_{50} value) and they kill their hosts faster (low LT_{50} value). In the field under sub optimal conditions strains that persist well and can successfully infect a host are said to be ecologically fit.

Bioassays performed may not be indicative of field environments as they are carried under conditions that favour fungal infections. Often field trials result in poor or inconsistent pest control emphasising the need to conduct bioassays using parameters that closely simulate field conditions (Butt & Goettel, 2000). More tolerance towards

UV radiation, resistance to desiccation and microbial attack and sufficient endogenous reserves to survive long periods in the absence of a suitable host or other nutrient source is seen in ecologically fit strains.

The Insect Host

The susceptibility of insect pests to entomogenous fungi is influenced by a complex array of physiological, behavioural and morphological factors including insect density, developmental stage, diet, ecdysis, basking, preening and injury caused by mechanical, chemical or microbial agents.

Stress: Stress has long been recognised as a factor that may increase the susceptibility of insects to entomopathogens (Steinhaus, 1958a, b; Vago, 1963). Crowding, nutrition, chemical pesticides and the environment make up stress factors. The host immune system is weakened by some chemical pesticides enhancing the efficacy of *M. anisopliae* (Hiromori & Nishigaki, 2001). Starved *Plutella xylostella* larvae were more susceptible than fed larvae to *Paecilomyces fumosoroseus* isolate 1576 but starved and fed larvae were similar in susceptibility to *P. fumosoroseus* isolate 4461 (Altre & Vandenberg, 2001). To improve the efficacy of fungal BCAs in vector control programmes a better understanding of stress-induced host susceptibility is needed.

Insect nutrition: The host diet plays an important role in successful infections. Insects maintained on artificial diet can be more susceptible to infection than those fed on natural diet (Boucias *et al.*, 1984; Goettel *et al.*, 1993). Compared to field collected insects, laboratory reared ones can be more susceptible (Bell & Hamalle, 1971). Well fed insects differ in their susceptibilities compared with starved ones (Milner & Soper, 1981).

Ecdysis and Developmental stage: The success of entomopathogenic fungi relies on developmental stage of the host insect. Adults can be the most susceptible while pupal stages are often the most resistant stage. Early instar nymphs are more susceptible than older stages. The young larvae of the European corn borer (*Ostrinia nubilalis*) were more susceptible to *B. bassiana* than older larvae (Feng *et al.*, 1985). Within adult stages there could also be differences in susceptibility between different sexes and forms such as the aphid alates and apterae (Oger & Latteur, 1985). Bioassay results may significantly be affected by the time of inoculation prior to ecdysis and the length of the intermoult period. The penetrating fungus may be removed prior to the colonization of the insect if moulting occurs shortly after inoculation (Vey & Fargues, 1977; Fargues & Rodriguez-Rueda, 1979). In contrast, Goettel (1988a) found that *Ae. aegypti* larvae were more susceptible to *T. cylindrosporum* during their mouting period. Reduction in the prevalence of infection due to loss of inoculum on exuviae is seen at high temperatures as high temperatures accelerate insect development and reduce the time between moults (Vestergaard *et al.*, 1995).

Insect density: Epizootics in insect populations are influenced by insect density. Higher density of insects increases the probability of an insect coming in contact with the fungal BCA directly or with infected individuals. Fatal doses of *B. bassiana* conidia could be transmitted from adult (*Delia radicum*) fly-to-fly of a series of atleast six flies (Meadow *et al.*, 2000).

Insect behaviour: The dispersal of fungal BCAs and the development of epizootics is influenced by insect behaviour. Grooming behaviour of termites helped in spreading the conidia of *M. anisopliae* among individual (Kramm *et al.*, 1982). Significant mortalities in the aphid population are induced by foraging coccinellids which transfer

conidia of *P. fumosoroseus* from sporulating cadavers to healthy aphids (Pell & Vandenberg, 2002).

Sublethal effects: Not all insects treated with a fungus succumb to infection. Studies on sublethal effects of entomopathogenic fungi are insufficient. The fecundity of Colorado potato beetle *Leptinotarsa decelimneata* was much lower in those surviving treatment than in beetles that were not treated (Fargues, *et al.*, 1991). Determination of sublethal effects needs to be explored further.

2.9 Host response to fungal invasion:

To prevent fungal invasion the defense mechanisms evolved by insects can be broadly classified in two groups:

1. Non-specific immunity: It consists of structural and passive barriers like cuticle, gut physicochemical properties, and peritrophic membrane (PM) (Chapman, 1998).

2. Specific immune system: It involves cellular and humoral immunity.

Cellular reactions include phagocytosis, nodulation (haemocyte aggregation) and encapsulation. Under humoral reactions activation of prophenoloxidase cascade and induction of immune proteins like lysozymes, lectins, production of protease inhibitors and anti-fungal proteins (Gillespie, *et al.*, 1997) are included.

Non-specific defence mechanism:

Morphological: The first line of defence is the chitinous cuticle of the insect covering the entire external surface, extending through the foregut, hindgut and tracheal tubes protecting the underlying epithelium. The insect midgut epithelium is not lined by cuticle (Wigglesworth, 1972). The cells of the midgut are apparently protected from injury due to hard or sharp food particles and from pathogens by the peritrophic membrane in many insects (Narayanan & Jayaraj, 1974).

Physiological: Behavioral responses maybe induced in an insect exposed to fungal spores, by the developing fungus in its host. Grasshoppers/locusts exhibit 'basking' wherein they elevate their body temperatures higher than ambient through habitat selection and/or orientation to solar radiations. By doing so they intercept the solar radiation and raise internal thoracic temperature ranging from 38°C to 42°C, thereby showing 'behavioural fever' response. This rise in body temperature is predicted to inhibit fungal proliferation, giving the host immune system an edge in suppressing the fungus germination and growth which normally takes place from 25°C to 30°C, thereby reducing the infection. Grasshopper inoculated with *B. bassiana*, in nymphs permitted to bask for only 1 h per day, 46% less mycosis was observed, and as the basking period increased, so did the inhibition of the disease. Through thermoregulatory behavior, in this study grasshoppers were able to cure themselves from mycosis (Inglis *et al.* 1996a).Thus, thermoregulation by grasshoppers, *M. sanguinipes* has been shown to reduce mycosis caused by *B. bassiana* and *M. anisopliae* (Ouedraogo *et al.*, 2003). Behavioral fever can be effective and is probably related to fungal optimum growth temperatures. The heat is thought to boost the host immune systems and retard fungal growth. When the thermal gradient was taken away, they quickly succumbed to infection (Elliot *et al.*, 2002). More success in eliminating insects was achieved by fungal spraying during cool overcast days compared to spraying in hot and sunny season (Inglis *et al.*, 1996a). The insect behavioral fever can only be effective if the environment provides a means for the insect to raise its temperature (through the sun).

Specific defense mechanism

Cellular immunity: Plasmocytes and granulocytes are the major effector cells amongst the six major groups of insect haemocytes in recognizing the 'self'

(isografts) and non-self (allografts). Foreign invaders like microorganisms are either phagocytosed by them or if the objects are too large to be individually engulfed, then they nodulate and encapsulate the object. Drastic reduction in the number of haemocytes during various microbial infections has also been reported by several workers. Infection by *B. bassiana* in *Spodoptera exigua* results in a gradual suppression of the phagocytic competence of circulating haemocytes, especially granular haemocytes, and alteration in total and differential haemocyte counts (Hung, *et al.*, 1993).

Humoral reactions: Fungal infection in insects elicits an acquired humoral "immunity" to subsequent infection. To initiate the haemocytic defense reaction in insects, recognition of "non-self" is necessary and this selective response depends on specific chemical recognition by the haemocytes. Serum and haemocyte cell membrane-bound lectins have been found in many insects (Mello, 1999). They could play a role in immune defense reactions as they agglutinate pathogens as well as fungi (Mello, 1999). Thus, insect serum agglutinin may opsonize mediating the enhanced attachment of granulocytes to the hyphal bodies (Pendland *et al., 1*988).

Melanization: Many insects respond to fungal infection by the production of brown or black melanic pigments, which is a result of the oxidation of phenolic compounds to dihydroxyphenyl- alanine. Phenoloxidase, an enzyme which is responsible for the melanization and encapsulation of pathogens in insects is synthesized by the phenoloxidase pathway. The 'sticky' nature of the activated phenoloxidase helps it in adhering to foreign particles and cytotoxic quinoid compounds are generated which kill the intruders (Ashida & Yoshida, 1988). Melanin may partially shield cuticle from enzymatic attack or may be toxic to fungi. However, such protection is incomplete. There is an indication that melanization is primarily an effective defence against weak or slow growing pathogens, but is ineffective against more virulent fungi (St. Leger,

1988a). Not much work has been carried out on the survival and development of insect mycopathogens within the host insect. Basic studies on the *in vivo* development of insect mycopathogens like white muscardine *B. bassiana*; green muscardine *M. anisopliae*, *N. rileyi* and *V. lecanii*, have focused on 'determinants' responsible for attachment, germination and cuticular penetration. The internal defence of the host might be overcome by the fungal pathogen utilizing one or more of the following strategies, the fungal cells developing within the insect maybe:

1. Possessing an outer coat, which is neutral to circulating haemocytes.
2. Effectively masked by host proteins.
3. Producing immunomodulating substances which suppress the cellular defence system. This renders them tolerance to the humoral and cellular defence system of the insects.

2.10 Application of the mycoinsecticides:

2.10.1 Bioassays of entomopathogenic fungi:

For the successful development of entomopathogenic fungi as biocontrol agents well designed bioassays are crucial. Bioassays can be used to determine and quantify host-pathogen relationships and the effect of biotic and abiotic parameters on these. They are the tools for identifying the key parameters like host range, virulence, comparison of virulence among isolates, ecological competency, conditions impeding/enhancing epizootics, barriers to infection and studies on effects of biotic and abiotic factors such as host age, temperature, humidity and formulation. The production, formulation and application methods employed can also influence fungal viability, virulence and efficacy. Bioassay techniques have been reviewed by Goettel & Inglis (1997), Kerwin & Peterson (1997) and Papierok & Hajek (1997). To develop a bioassay protocol a

thorough understanding of both the pathogen and host requirements is essential. Otherwise it will lead to inconsistent results, high mortality and poor assessment of fungal virulence. The objective of a bioassay must be well defined and designed to address these objectives and provide meaningful results. Choosing the host developmental stage, rearing the host, infective propagule, formulation and inoculation method, conditions of post-inoculation incubation, method of mortality assessment (including mortality in controls), bioassay design and statistical analyses must be carefully considered.

A non-treatment control is to be included in all bioassays in order to monitor survival of insects under the post-inoculation incubation conditions. If a carrier has been used for application of inoculum then the control insects also should be treated with the carrier. If bioassays are used for host range or safety to non-target organisms, it is essential that a known susceptible host is also treated with the non-target organisms (i.e. positive control).

Choice of sample size and dose range determination is an important aspect of a bioassay. From the results of preliminary bioassays carried using a wide range of doses and relatively small number of hosts, those doses which result in mortalities between 25-75% must be chosen.

An inoculation method should ensure presentation of a precise dose which will reduce variability and help ensure repeatable results. Also the size and fragility of insect and the form of inoculum are important. Details on enumeration of propagules are presented by Goettel & Inglis (1997) for Hyphomycetes, Kerwin & Peterson (1997) for water moulds, and Papierok & Hajek (1997) for Entomophthorales. In case of terrestrial insects the inoculum can be introduced *per os* or directly into the

haemocoel by piercing the intersegmental membrane. Aquatic insects are treated by introducing known numbers of propagules into their rearing medium.

Bioassay chambers are chosen according to the price, availability, convenience, ease of cleaning and requirements of the host. It is important to decontaminate the chambers prior to reuse or alternatively disposable containers can be used. Choice of bioassay chamber is critical in field-cage bioassays. Inglis *et al.* (1997a) found the differences in temperature and relative humidity within and outside the cages minimal, but there was approximately 55% shading within the cage due to the mesh screening. After inoculation the insects should be incubated under controlled environmental conditions. Mortality assessments should be made daily and median lethal doses calculated which will help in comparison between different treatments.

2.10.2 Factors affecting the virulence of mycoinsecticide

Factors like solar radiation, temperature, relative humidity, precipitation and wind govern the success of entomogenous fungi against insects.

1. Solar Radiation

Propagule persistence in epigeal habitats is influenced by solar radiation (Moore *et al.*, 1993; Farques' *et al.*, 1996; Smits *et al.*, 1996a, b). The fungal propagules are highly susceptible to damage by UVB (280-320 nm) and to a lesser extent UVA (320-400 nm). Braga *et al.* (2001) showed that the culturability of conidia of different strains of *M. anisopliae* reduced by 10-60% on 4h exposure to solar UVA. *B. bassiana* conidia are almost completely inactivated by exposure to 1h of direct sunlight or 20s of UVB (Edgington *et al.*, 2000). To protect conidia from harmful radiation solar and UV blockers are often incorporated into formulations (Burges, 1998). Lacing the conidia of *B. bassiana*, *M. anisopliae* and *M. anisopliae* var. *acridum* with oils or oil-soluble sunscreens showed s ignificant increase in survival when exposed to artificial radiation

(Moore *et al.*, 1993; Inglis *et al.*, 1995a; Alves *et al.*, 1998) but they did not enhance survival in field settings (Inglis *et al.*, 1995a). In contrast a solar blocker (clay) and a UV absorbing optical brightener (Tinopal) did increase the field persistence of *B. bassiana* conidia on grass leaves exposed to sunlight (Inglis *et al.*, 1995b).

2. Temperature

The efficacy of entomogenous fungi is affected by temperature considerably (Inglis *et al.*, 2001). The optimum temperature of entomogenous fungi is between 20-25°C in laboratory based bioassays, being infectitious over a wide range of temperature (10-30 °C). Vegetative growth of most genera is inhibited above 30°C, and growth usually ceases at 37 °C. In the risk assessment and registration of Fungal Biocontrol Agents (BCA) the inability of an entomopathogenic fungus to grow at mammalian body temperature is an important consideration. There exists considerable variability among genotypes in their thermal characteristics (Fargues *et al.*, 1997b) with little or no correlation between the geographic origin of the strains and their thermal properties (McCammon & Rath, 1994; Fargues *et al.*, 1997a; Ouedraogo *et al.*, 1997; Vidal *et al.*, 1997). Temperatures which favour spore germination may not be optimal for growth. In *B. bassiana* temperature had a significant effect on both germination rate and vegetative growth. The fastest germination occurred at 25-32 °C and the fastest growth occurred at 30 °C (James *et al.*, 1998). The timing of application of inoculum may be crucial to the successful deployment of fungal BCAs as low night temperature may reduce the percentage germination.

3. Relative humidity (RH)

At all stages of lifecycle of entomopathogenic fungi water is critical (spore germination, conidiogenesis at the surface of mycosed cadavers). Hence the efficacy of entomogenous fungi including recycling and transmission within pest populations

can be profoundly influence by the ambient relative humidity (Milner & Lutton, 1986; Heyler *et al.*, 1992; Fargues & Luz, 2000). Humidities greater than 96% are required for spore germination and mycelial growth (Ibrahim *et al.*, 1999) by most fungi. *B. bassiana* for production of conidia on mummified cadavers of *Rhodnius prolixus* required a RH of at least 97%. In the mycosed cadavers of *Schistocerca gregaria* sporulation of *M. anisopliae* var. *acridum* is a dynamic process dependent on the water content of the locust (Arthurs & Thomas, 2001). Certain studies have shown that dry conditions during or following the application of fungal propagules are less detrimental than previously thought. As in *B. bassiana* and *M. anisopliae* var. *acridum* which are able to infect their respective hosts under conditions of low ambient humidity presumably due to sufficient moisture within the microhabitats such as intersegmental folds of the insect integument.

Compared to water based formulations, oil-based formulations appear to be superior for the control of arthropod pests (Burges, 1998; Prior *et al.*, 1988). Oils presumably decrease the dependency on saturated conditions normally required for successful infections (Bateman *et al.*, 1993). Over the insect surface they flow readily, carrying the spores to sites that are conducive for germination and infection such as the intersegmental membranes (Ibrahim *et al.*, 1999).

Rainfall

Rainfall increases humidity. Within a relatively short period of time it can dislodge and dispose conidia from plant and insect surfaces (Inglis *et al.*, 2000; Inyang *et al.*, 2000). New formulation and application strategies ensure better retention of conidia on leaf surfaces especially the underside of leaves where most foliar pests concentrate. Oil carriers appear to offer greater rainfastness than aqueous carriers (Inglis *et al.*, 2000; Wraight & Ramos, 2002). Conidia in direct contact with the

leaves and cuticle of beetle larvae were less prone to be removed by rain than the aggregates of conidia which made little contact with the conidia (Inglis *et al.*, 2000).

Soil

The persistence and efficacy of fungal BCAs is influenced by the soil which is an extremely complex environment with several interacting factors (Studdert & Kaya, 1990a, b). These factors include soil type (i.e. texture, cation-exchange capacity, organic matter content, pH, water-holding capacity, etc.) and soil biota (i.e organisms that inhibit or feed on conidia). Various formulations (conidia powder, blastospores, lyophilised mycelium, inoculated kernels) and applications (drilling, spraying, injection) can influence the success of entomogenous fungi in the soil. In temperate climates, conidia persist well whereas the thin-walled blastospores are comparatively short-lived (Gaugler *et al.*, 1989; Storey *et al.*, 1989; Inglis *et al.*, 1997b; Keller *et al.*, 2000).

The distribution and efficacy of the major entomopathogenic species including *M. anisopliae*, *B. bassiana*, *P. farinosus*, *P. fumosoroseus*, *V. lecanii* and *Tolypocladium* spp. appears to be affected by soil texture (Vanninen, 1996; Chandler *et al.*, 1997; Bidochka *et al.*, 1998).

2.11 Bio-safety of non target organisms:

Vertebrates, invertebrates and plants not intentionally being affected by the biological control fungus are referred to as the non target organisms (NTO). For biological control using fungi, the bio-safety of the microorganisms being contemplated for use must be considered at many levels. Concerning the safety of NTOs the following issues have been identified i) Competitive displacement of NTOs ii) Allerginicity iii) Toxigenicity to NTOs iv) Pathogenicity to NTOs (Cook *et al.*, 1996). Other indirect

safety issues that could arise due to the depletion of the target host itself, must also be considered as a potential safety issue (Goettel & Hajek, 2000). Safety of biocontrol fungi has been extensively reviewed (Austwick, 1980; Goettel *et al.*, 1990; Evans, 1998; 2000). Flexner *et al.*, 1986; Laird *et al.*, 1990; Cook *et al.*, 1996; Goettel & Jaronski, 1997; have reviewed safety of microbial control agents. Guidelines for testing pathogenicity and infectivity of entomopathogens to mammals have been reviewed by Siegel (1997) and guidelines for evaluating effects of entomopathogens on invertebrate non-target organisms have been reviewed by Hajek & Goettel (2000).

Competitive displacement of non-target organisms:

In an ecosystem the fungal biocontrol agents introduced may competitively occupy a niche adversely affecting one or more native organisms within that niche which might be an intended effect. But if a non-target organism is competitively displaced leading to its extinction or affecting some other component of the ecosystem it would be a fatal unintended effect. Fungal pathogens and insect parasitoids may compete within the host tissues (Goettel *et al.*, 1990; Vinson, 1990). The older parasitoids are often capable of completing their development within fungus-infected insects while the fungus usually out-competes the younger parasitoids.

Allergenicity:

Fungi are capable of producing spores that cause allergies or allergic reactions. The actual or potential fungal biocontrol agents are not amongst the species that are responsible for the production of common allergens (Latge & Paris, 1991). However allergic reactions have been reported by those exposed during mass production and application of microbial control fungi (Austwick, 1980). *B. bassiana* has been reported to cause allergies in humans (York, 1958) and is an opportunistic pathogen to man and other mammals (Burges, 1981) and is registered as a dermal sensitizer (Saik

et al., 1990) by the US Environmental Protection Agency (EPA). Unprotected humans should not be exposed during production and application as all fungi are potentially allergenic.

Toxicity:

A wide variety of compounds with biological activity against other organisms mostly products of secondary metabolism are secreted by fungi. The first systematic *in vitro* study of toxin production by fungal entomopathogens was conducted on *M. anisopliae* and led to the discovery of destruxins (DTX) A and B (Kodaira, 1961). Destruxins can be toxic by injection, ingestion and/or topical application, depending on variant and host, Lepidoptera and adult Diptera are particularly susceptible. DTX exhibits toxicity towards vertebrates (Debeaupuis & Lafont, 1985; Genthner *et al.*, 1998). The mite pathogen *Hirsutella thompsonii* produces Hirsutellin A that is toxic by contact to its host, the citrus rust mite *Phyllocoptruta oleivora* (Omoto & McCoy, 1998).

B. bassiana produces oosporein, cyclosporins, bassianolide and beauverolide. An antiparasitic and antifungal property, as well as immunosuppressive and anti-inflammatory activity in humans is exhibited by Cyclosporins which are cyclic undecapeptides (Wartburg & Traber, 1988). They were originally isolated from *Trichoderma polysporum* (Ruegger *et al.*, 1976) and more recently from *Tolypocladium cylindrosporum, B. brogniartii, B. nivea* and *Verticillium* sp. Cyclosporins A, B, and C have insecticidal properties (Weiser & Matha, 1988a).

Safety of extracellular metabolites has been tested against *Daphnia pulex, Cyclopes, Lymnea auriculeta,* and tadpoles of *Rana tigrina* (Vyas *et al.* 2007). Before using a toxin producing fungal BCA several considerations are to be made like possible effects from the presence of the toxins in the formulated product (e.g. danger to applicator), fate of the toxin after application like the rate of degradation and possible bioaccumulation of the toxin within the host or the environment (e. g. danger

to scavengers feeding on cadavers). In the case of *Fusarium nygamai* which was considered as a promising mycoherbicide for control of witch-weed (*Striga hermonthica*) is now shown to produce mycotoxins including several novel compounds (Capasso *et al.*, 1996) which are a potential threat to vertebrates.

Pathogenicity:

Pathogenicity towards the target host is the desired effect. Entomopathogenic fungi can infect a wide variety of hosts which are non target organisms. High mortality appeared in non-target invertebrates, when they contacted or ingested spores of the entomopathogenic fungi. Larvae of the coccinellid *Cryptolaemus montrouzieri* suffered 50% mortality when fed Boverin-t, a commercial conidiospore preparation of *B. bassiana* (Flexner *et al.*, 1986). Honey bee workers experienced 29% mortality when fed spores of *H. thompsonii* (Cantwell & Lehnert, 1979). Both *B. bassiana* and *M. anisopliae* infect *Bombyx mori* and also killed honey bees following field applications (Podgwaite, 1986).

Captive American alligators and a Giant Tortoise that had been stressed by chilling were reported to be infected by *B. bassiana* (Saik *et al.*, 1990; Semalulu *et al.*, 1992). Both embryos and newly hatched larvae of the inland silverside fish were adversely affected by *M. anisopliae* conidia (Genthner & Middaugh, 1995). Effects included decreased cardiac output, chorionic rupture and teratogenic expressions in embryos and larvae. In both immunocompetent and immunoincompetent individuals, cases of human infection by the same fungus (Burgner *et al.*, 1998; Revankar *et al.*, 1999) and *Paecilomyces lilacinus* (Itin *et al.*, 1998; Gutierrez-Rodero *et al.*, 1999) have been reported. *Conidiobolus coronatus* is associated with lesions in humans and horses (Saik *et al.*, 1990). To determine the virulence of fungal BCAs towards NTOs with special consideration to human exposure scenarios tests should be conducted.

No infectivity to man or other vertebrates was seen in most of the epf developed for commercial use in microbial control of insect pests (El-Kady *et al.*, 1983; Podgwaite, 1986). Safety tests with *N. rileyi* (Ignoffo, 1973; Ignoffo, *et al.*, 1979), *H. thompsonii* (McCoy & Heimpel, 1980), *V. lecanii* (Podgwaite, 1986) and *L. giganteum* (Kerwin, *et al.*, 1990) did not show negative effects against different mammals and birds (Kerwin, 1992).

Depletion of hosts:

Reduction in the target host population may affect other organisms that depend on this pest/vector. The extent and the speed of host depletion and the length of time the host has been in the targeted location determines the degree of harm to the NTO. When the Australian pathotype (*Entomophaga praxibuli*) of the grasshopper-pathogenic fungus *Entomophaga grylli* was introduced into the US, Lockwood (1993) speculated that suppression or even extinction of the target as well as non-target acridids may result in the loss of biodiversity. Further it may cause proliferation of new weed species, disruption of plant community structure, suppression of essential organisms vectored by grasshoppers and disruption of food chains and other nutrient cycling processes. In rebuttal Carruthers & Onsager (1993) pointed out that endemic *E. grylli* pathotypes 1 and 2 already periodically produce epizootics in grasshopper populations and reduce outbreak of grasshopper populations. In practice, release of the Australian pathotype was made in North Dakota and Alaska. Despite initial evidence that the fungus established and increased in prevalence (Carruthers & Onsager, 1993), current evidence suggests that the frequency of infection has reduced to levels such that long-term survival of this pathotype in North Dakota is questionable (Bidochka *et al.*, 1996).

Fungi are tested in 5 tiers in the US and those that clear the 1st tier are approved for sale as in the case of *L. giganteum* (Kerwin *et al.*, 1994), *V. lecanii* (Lisansky & Hall, 1983), and *H. thompsonii* (Ignoffo *et al.*, 1973).

2.12 Regulations and Commercial formulations:

2.12.1 Regulation of entomopathogenic fungi as microbial pesticides:

Regulations and registration requirements alleviate potential safety issues and ensure safety of the agent and its efficacy. The regulatory authorities have to take care that the research is not slowed down and the development and implementation of microbial control is not impeded. The data requirements of different countries differ for registration of a microbial control pesticide agent (MCPA). A company wishing to register its product in several countries will have to give different registration packages thereby significantly increasing the costs of registration of products which are best for niche markets generally (OECD, 1996). WHO proposed a tiered testing strategy to assess the hazard posed by MCPA to mammals (Anon., 1981). Elements of this strategy are currently included in the regulatory guidelines of Canada, USA, the European Union. The tests replaced the long-term assays required for chemical pesticides with short-term (1 month exposures utilizing invasive routes (intravenous and /or intraperitoneal injections) as well as feeding studies. This battery of tests is referred to as Tier 1. *Lagenidium giganteum* (Kerwin *et al.* 1994), *Hirsutella thompsonii* (Ignoffo *et al.*, 1973), *Nomuraea rileyi* (Ignoffo, 1981) and *Verticillium lecanii* (Lisansky & Hall, 1983) are amongst the Fungi that cleared first tier of tests and are approved for sale in the US.

A microbial control agent will have to undergo more extensive Tier 2 testing if some doubts arise during Tier 1. Usually a MCPA which does not make it in Tier 2 is not

considered a viable control product and may not undergo Tier 3 testing which is also available. The data from such tests is unavailable to public as it is considered proprietary except in the form of results in peer reviewed publications as published by Burges (1981), Saik *et al.* (1990), Semalulu *et al.* (1992), Goettel & Jaronski (1997), Smits *et al.* (1999). The perspective for handling safety data and field reports was lucidly given by Burges, "A no risk situation does not exist certainly not with chemical pesticides and even with biological control agents one cannot absolutely prove a negative. Registration of a chemical is essentially a statement of usage and the same should be applied to biological agents." Registration requirements for biocontrol fungi must remain flexible and address the hazards of the specific candidate and its proposed use on a case by case basis (Goettel *et al.*, 2001).

2.12.2 Commercial formulations:

EPF must be formulated in a manner to ensure its application with aid of simple agrochemical tools. Live propagules with maximum virulence are mass produced which is unique in fungal insecticides. Standardisation of all forms to tolerate extremities of temperature, moisture and aeration until use is carried out (Lisansky & Hall, 1983).

Surface culture: This technique involves either a surface or a two-stage cultivation process. Abundant conidia are obtained by these methods. Using agar, cereals and a variety of solid wastes fungi can be grown in solid substrate fermentation. Expensive agar media and longer metabolism time required for solid wastes make cereal grains the preferred raw material. Inoculum levels have to be high enough to lower fermentation time but not cause early depletion of nutrients. Light or near-UV irradiation induces sporulation. The solid substrate cultivation is however time consuming, labour intensive and uneconomical. Under submerged conditions the

fungus is first grown and then allowed to sporulate as a surface culture in still liquid or semi-solid medium in the two-stage cultivation process (Hall & Papierok, 1982; Roberts & Sweeney, 1982). This problem can be overcome by submerged fermentation.

Submerged fermentation: The ability to modify and regulate substrate quality and quantity, temperature, aeration, mixing, pH, light and timing of sporulation makes submerged fermentation a preferred method for large-scale production of any microorganism. Continuous culture selection to select strains compatible with chemical insecticides and fungicides, strains of same organism with different temperature optima and biochemical components can be produced by this method. The drawback is most fungi imperfecti do not produce conidia in submerged culture, but produce mycelia and blastospores in abundance (Samsinakova, 1966; Roberts & Sweeney, 1982; Latge & Moletta, 1988; Jenkins & Prior, 1993). Unlike conidia the latter although infectious, are short-lived and cannot withstand adverse environmental conditions (Van Winkelhoff & McCoy, 1984; Ferron, 1978; Adamek, 1965; Roberts & Sweeney, 1982). *Metarhizium flavoviride* has been grown in a simple liquid medium by altering conditions, producing sporogenous cells and spores morphologically similar to that of aerially grown fungus (Jenkins & Prior, 1993). Careful control of nutritional requirement in the liquid culture medium resulted in desiccation tolerant blastospores of *P. fumosoroseus* (Jackson *et al.*, 1997). In practice conidia of *B. bassiana* were produced by this method in the erstwhile USSR and the cultivation process has been patented (Goral, 1971; Kondryatiev *et al.*, 1971). Thomas *et al.* (1987) have reported a method and medium to produce conidia of *B. bassiana* under submerged cultivation and compared the characteristics of the conidia with that grown on solid substrate. The stability and virulence of conidia of this fungus

produced under submerged cultivation is known to some extent (Lisansky & Hall, 1983) but very little is known about the cultivation process or the factors regulating conidiation.

Processing and formulations: After determining optimum drying rates and desired moisture levels for each fungus the spores are formulated to stabilise them during storage and facilitate their application. The formulation has to have a long shelf life and should be capable of being stored up to 18 months (Couch & Ignoffo, 1981) to sustain market demands. Addition of wetters, stickers, dispersants, UV protectants etc., enables easy and effective delivery at target sites and maintains its *in situ* activity (Lisansky & Hall, 1983). In the field conidia, mycelial fragments or blastospores are applied in the form of aqueous solutions (Li *et al.*, 1993; Butt *et al.*, 1994). The stability, sensitivity and persistence of formulations is influenced by abiotic factors like sunlight, temperature, humidity, substratum and chemical pesticides, either independently or collectively influence (Ibrahim *et al.*, 1999).

Successful control of some agricultural pests has been achieved using oil-based formulations (Prior *et al.*, 1988). Infections at lower humidities (Bateman *et al.*, 1993), stimulated germination (Winder & Van Dyke, 1990), longer duration of viability (Prior *et al.*, 1988), decreased sensitivity to high temperature during storage (McClatchie *et al.*, 1994), decreased sensitivity to UV radiation (Moore *et al.*, 1993) and enhanced attachment to hydrophobic surface of insect integument (Inglis *et al.*, 1996b) is seen in oil based formulations.

The viability of microbial cells is retained water loss is slower during drying process. Physical microbial cell damage is lesser when dried at high humidities and allows intracellular accumulation of acyclic sugar alcohols and other osmoprotectants (Hallsworth & Magan, 1994). To increase the shelf life of the fungal preparations

certain sugars, chitin and starch are added providing physical stabilization and osmoprotection during desiccation and nutritional sources during rehydration (McCabe & Soper, 1985; Li *et al.*, 1993; Pell *et al.*, 1998; Shah *et al.*, 1999). Mycelial mats of *B. bassiana* and *M. anisopliae* could be stored at 4 °C, up to 3 months (Periera & Roberts, 1990). Alginated mycelia of *B. bassiana* could be stored for 3-5 months at room temperature whereas *M. anisopliae* required 4 °C and was sensitive to temperature (Knudsen *et al.*, 1990; Pereira & Roberts, 1991). Compared to mycelia, hyphal cells of *E. neoaphidis and Zoopthora radicans* infecting insect cadavers indicated greater storage potential than mycelium produced *in vitro* (Pell & Wilding, 1992).

2.13 Persistence and recycling of mycoinsecticides in the environment:

Wind and water are the most important abiotic factors that help in spore dispersal in most Hyphomycetous fungi as they do not possess any specific mechanism for spore dispersal. In the Oomycetes the spores are motile and reliant on the presence of water. Spores are forcibly discharged and land centimetres away from the host or can be carried over longer distances by air currents in Entomophthorales. Biotic factors include movement of humans and animals through the treated areas, predation of sporulating cadavers and the emigration of infected insects. Also the ability of the fungus to sporulate from infected cadavers helps in spore dispersal.

As most spore types are very sensitive to ultraviolet radiation, spores exposed to sunlight are short lived. Persistence is generally much increased in shaded habitats and soil. In contrast, the thick walled resting spores produced by species within Oomycetes, Zygomycetes and some Ascomycetous fungi are capable of persisting for several years under adverse abiotic conditions.

L. giganteum persisted in the absence of mosquito larvae under favourable temperature and water conditions (Jaronski & Axtell, 1983). It was concluded by them that the fungus could persist at least for an entire mosquito breeding season, as a self-perpetuating biocontrol agent of mosquito larvae. The seasonal appearance of *Culicinomyces clavisporous* was attributed to recycling of the fungus by rainfall and flooding of rock-pools with river water. Its conidia could persist and remain infective and this period could be increased by lowering the temperature (Frances *et al.*, 1984, 1985).

2.14 Integrated use of mycoinsecticides in vector management:

Entomopathogenic Hyphomycetes have been applied using inundative application or microbial pesticide method in epigeal environments where as in soil environments inundative as well as inoculative strategies are used. Ground and aerial applications of mosquito pathogenic fungus *L. giganteum* was made in rice fields and associated habitats in California. It resulted in greater than 90% initial mortality of *Cx. tarsalis* and *An. freeborni* larvae and 65% in *Ae. melanimon* larvae accompanied by a 10-fold decrease in indigenous populations of the former two (Kerwin & Washino, 1987). Convergence is needed between integrated pest management and integrated vector management strategies to help farmers improve their agricultural practices while minimizing environmental risks to health. In mosquito pest management programs Agra Quest has developed *L. giganteum* as LAGINEX providing long term control against the larval stage of mosquitoes. No adverse effects against other insects, mammals, fish, birds, or plants are recorded (www.agraquest.com). It controls larvae of *Aedes*, *Anopheles*, *Coquillettidea*, *Culex*, *Culiseta, Deinocerites, Eretmapodites, Haemagogus, Mansonia, Opifex, Orthopodomyia, Psorophora, Sabethes,*

Uranotaenia and *Wyeomyia* genera in water sources with good water quality. LAGINEX is registered for use on rice, soybeans and irrigated pasture. It's effectiveness at temperature $<32^0$C limits its use. The Bio-Path® Cockroach Control Chamber uses *M. anisopliae* as the microbial agent. The fungus has been marketed as safe for use in hotels, restaurants and museums. There is increasing interest in biological methods having sublethal and lethal effects at different ages and stages in the mosquito life cycle rather than causing instant vector mortality for vector control. Integration with other vector control interventions, may produce substantial reductions in disease transmission as an overall effect of alterations at relevant points to multiple life history parameters and transmission-cycle of the vector. Biopesticides containing a fungus that is pathogenic to mosquitoes may be an effective means of reducing malaria transmission, particularly if used in combination with insecticide-treated ITNs. Significant reductions in the longevity of adult *Anopheles* mosquitoes, killing them within 14 days was shown by using *M. anisopliae* and *B. bassiana isolates* by Scholte *et al.* (2003b, 2005) and Blanford *et al.* (2005). The selection pressure for fungal resistance development is reduced by slow speed of kill (Thomas & Read, 2007; Read *et al.*, 2009). Delayed kill adds up to is reducing the mosquito's vectorial capacity as *Plasmodium* maturation also takes 14 days. Reductions in *Plasmodium* sporozoite formation (Blanford *et al.*, 2005), feeding propensity (Blanford *et al.*, 2005, Scholte *et al.*, 2006), and fecundity (Scholte *et al.*, 2006), are a few prelethal effects of fungal infection, further increasing the potential impact on malaria transmission.

Chapter 3

MATERIALS AND METHODS

"Art and science have their meeting point in method"

3.1 Mosquito Culture:

3.1.1 Adult rearing: To ensure a continuous supply of larvae of the three species the adult mosquitoes were collected from human habitats, identified and released in separate cages specially designed for them in the insectary. The cubical cages of 2 ft^3dimension were made of nylon net and cotton cloth hung over a metallic framework by corner loops (Fig. 3.a). The mosquito cage had circular front opening of ~1 ft diameter which was large enough for inserting an iron fowl cage, transfer of mosquitoes, etc. Usually, this opening was kept tied to prevent the adults from escaping. Many such cages were used to rear three test mosquito species viz., *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*.

The field collection of the immature forms of mosquitoes from their breeding habitats was also carried out following the methods of Service (1976).

3.1.2 Rearing of immature stages of mosquito:

From the insectary of National Institute of Malaria Research, Field Station, Panaji, Goa, 3rd instar larvae of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, used in the present study, were obtained. The cyclic culture of mosquito life stages were maintained in the table-top insectary with the help of trained insect collectors, technicians and lab assistants. The larvae were maintained at a temperature of 25 $\pm$ 2°C and RH of 70 $\pm$ 5%. Larvae were reared in round plastic basins of 11 inch diameter on the top using aerated tap water (Fig. 3.b).

3.1.3 Mosquito food:

20-25 mg farex was provided as larval feed every 24 hours in each of the larval rearing basins. A live chicken was introduced every 48 hours in the adult cages in the iron frames to provide blood meal to the adult females. Petri plates containing raisins

Fig. 3 Table-top insectary at NIMR, Panaji, Goa. **a.** Mosquito cages **b.** Mosquito cultures

kept on cotton pads soaked in 5% glucose in the cages served as a sugar source for the adult mosquitoes especially the males.

3.1.4 Egg collection:

The gravid female mosquitoes oviposited in the plastic bowls containing 250 ml tap water that were maintained inside the adult cages. The eggs were gently transferred along with water or with the help of soft brush in plastic basins containing dechlorinated tap water where they hatched. When these larvae grew to 3^{rd} instars in about 4 days they were used in bioassays or were further reared to 4^{th} instar, pupae and finally adults to maintain the running culture of the test species in the insectary as discussed above.

3.1.5 Aspirator/suction tube:

To capture adult mosquitoes, an aspirator/suction tube was used. It consisted of approximately 15 inches long plastic tube attached to a flexible rubber tubing about 20 inches long and a small plastic or glass mouth-piece. A fine wire mesh/guaze separated the plastic and rubber tube to prevent mosquitoes from entering into the mouth while suction/collection.

3.2 Sourcing of fungi pathogenic to insects:

3.2.1 Sourcing from nature:

Weather Conditions in the study area

Goa is a part of the coastal belt known as Konkan on the west coast of India. The ambient temperature at the collection sites ranged from 22-35°C. Mean annual rainfall was 200-350 cm and humidity ranged from 51-95% (Sourced from Met. Dept., Panaji, Goa).

Sample collection

Insect cadavers, live and dead mosquito larvae from breeding sites and dead adult mosquitoes (Fig. 4.a-d and Fig. 4.f, g) were collected from 4 different locations in Goa viz., Panaji, Taleigao, Ribandar and Farmagudi. From rice fields at Banastarim (Fig. 4.e) and backwaters at Carambolim, fungi were isolated using mosquito larval baits in simulation float chamber (Fig. 4.h) as explained in 3.2.2.

3.2.2 Sample isolation and processing:

Field isolation

Aquatic fungi were isolated using 3rd instar larvae of *Culex*, *Anopheles* and *Aedes* as baits (Kerwin and Peterson, 1997). A 500 ml plastic bowl with surface diameter of 12 cms and having two side windows of 2 cm x 2 cm dimension cut out 2 cms above the base of the bowl and fitted with nylon mesh served as the simulation float chamber. Ten 3rd instar larvae were introduced in 100 ml of water in the bowl. Then the bowl holding larvae was inserted in a round hole of 11 cm diameter cut out neatly in a 10 sq. inch size and ~ 1 inch thick wooden floatation plank such that when floating in water the plank would keep the mouth of the bowl above the surface of water. To prevent predation of mosquito larvae, the bowl was covered with nylon net and held with a rubber band outside the bowl circumference. Such floats were suspended in backwaters, rice fields, temporary puddles, etc. for a period of 3-5 days and the plank was held in its position with the help of a rope on one end and stationary objects on the other end. After 5 days, the bowls were taken out and the live and dead larvae or cuticle were transferred to a bowl with aerated tap water. The mouth of the bowl was covered by a net. A hole of 2 cm diameter was made in the centre of the net to enable

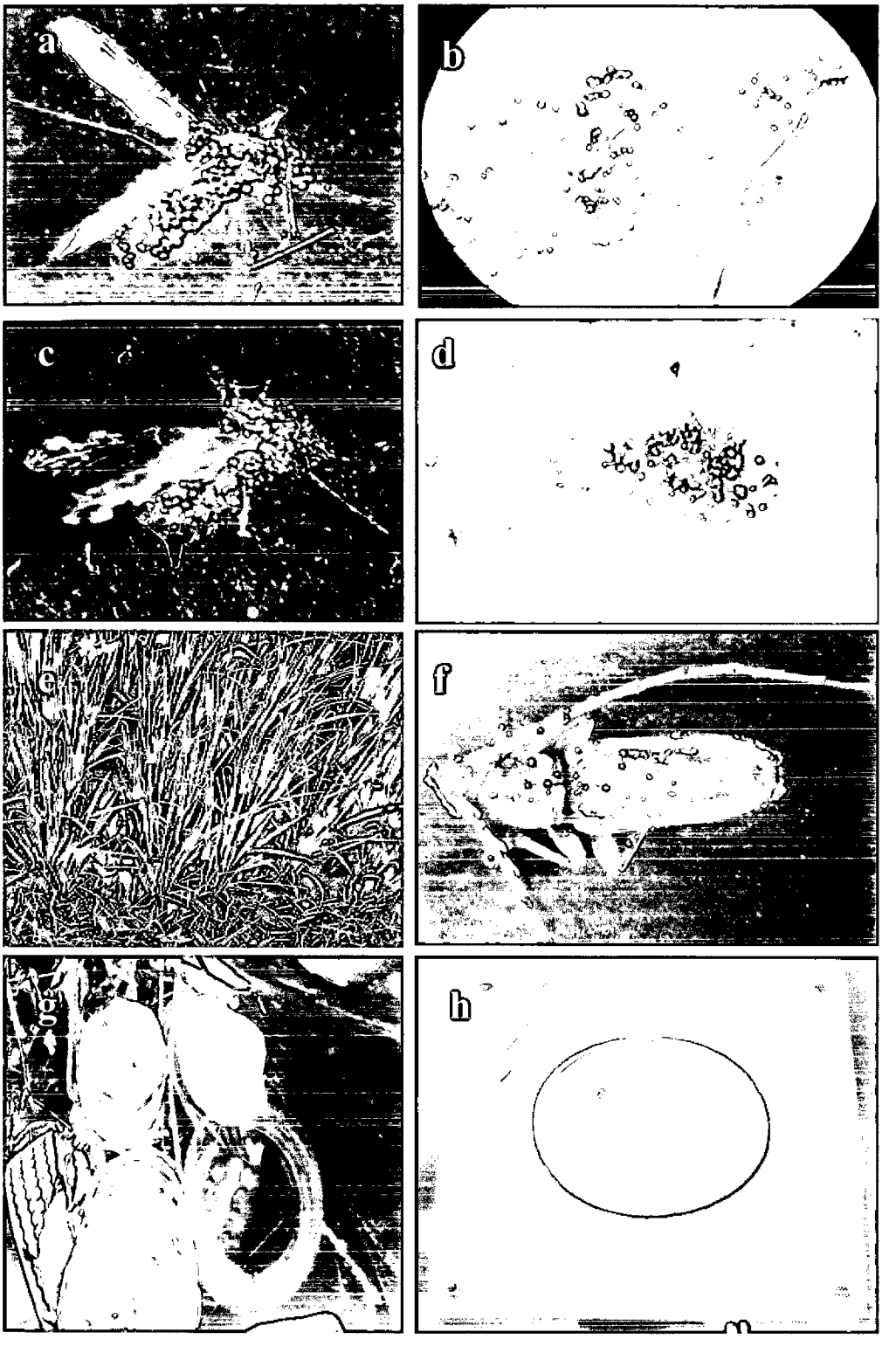


Fig. 4 Sources of Fungi: **a.** Adult of *Anopheles* sp. infected with *Aspergillus* sp. from NIMR lab, Panaji,16X; **b**. Infected Grasshopper from Farmagudi,16X; **c.** *Aspergillus* sp. growing on adult of *Anopheles* sp. from NIMR lab, Panaji,10X; **d.** *Cx. quinquefasciatus* larva infected with *Aspergillus* sp. from NIMR lab, Panaji, 16X; **e.** Rice fields at Banastarim a source of mosquito larvae **f.** Long horned beetle infected with fungus from Farmagudi,10X; **g**. Collection of mosquito larvae from tyres at Ribandar jetty **h.** Simulation float chamber with larval bait .

insertion of a dropper to pick up larvae. The hole was plugged by cotton. The picked immature were washed with water and surface sterilized by placing them in dilute sodium hypochlorite solution (1% NaClO) for 60 seconds followed by rinsing in 2-3 changes of distilled water. It was then blotted dry with sterile filter paper. The sluggish or dead larvae were observed under a stereoscope (Zeiss Stemi 1000) and a light microscope (Olympus CH30) for signs of fungal infection. If infected the material was streaked on appropriate medium supplemented with antibiotics.

Direct isolation

Collection of dead adult mosquitoes belonging to *Cx. quinquefasciatus, An. stephensi* and *Ae. aegypti* was carried out with the help of an aspirator from the mosquito cages in the insectary. In cadavers where already sporulation had occurred, direct isolation was done by lifting the conidia with a sterile needle tip and plating it on nutrient medium such as CMA and PDA supplemented with antibiotics (Goettel & Inglis, 1997).

Moist Chamber Technique

A 90mm Petri plate with a wad of moistened cotton covered with filter paper was sterilized and used as a moist chamber. Surface sterilized insects were placed in it and the Petri plate was sealed with Para film. In conditions of high humidity (80-90%) at 25°C sporulation was observed on the insect integument within 2-5 days. The spores were then transferred to the agar medium.

Isolation from insect cadavers

The cadavers were washed with sterile distilled water twice and their surface sterilization was done by placing them in dilute sodium hypochlorite solution (1% NaClO) for 60-90 seconds depending on their size, followed by rinsing in 2-3 changes of distilled water. After blotting with sterile filter paper they were streaked on sector

plates containing medium supplemented with antibiotics. MEA or PDA medium was used for isolation of few aquatic fungi and CMA medium was used for isolation of aero-aquatic hyphomycetes and entomopathogenic fungi.

3.2.3 Culture media:

For isolation of fungi Malt extract (5%) agar (MEA), Potato dextrose (4.1%) agar (PDA) and Corn meal (1.7%) agar (CMA) media were used. After autoclaving the medium an antibiotic cocktail at the concentration of bacitracin 20 mgL^{-1}, neomycin 20 mgL^{-1} penicillin G 20 mgL^{-1}, polymixin 20 mgL^{-1}, streptomycin 20 mgL^{-1} and tetracycline

20 mgL^{-1} dissolved in 10 ml of sterile distilled water was added to 1 L of medium to prevent bacterial growth in Petri plates during isolation.

a. Corn Meal Agar (CMA) medium

Seventeen gram of Corn Meal Agar, (Himedia Laboratories Pvt. Ltd.) was boiled in 1000 ml distilled water to dissolve completely and then sterilized in an autoclave at 121°C for 15 minutes. The ingredients contained in it were Corn meal infusion- 50 gL^{-1}; Agar-15 gL^{-1} with a final pH of 6.0 ± 0.2.

b. Malt Extract Agar (MEA) medium

Fifty gram of dehydrated malt extract (Himedia Laboratories Pvt. Ltd.) and 20 g of agar (E Merck India Ltd.) were dissolved in 1000 ml of distilled water by boiling and the pH was adjusted to 6.0 and sterilized in an autoclave.

c. Potato Dextrose Agar (PDA) medium

Potato dextrose agar 41gL^{-1} (MO96A, Himedia Laboratories Pvt. Ltd.) was dissolved in 1000 ml of distilled water by boiling and sterilizing in an autoclave at 121°C for 15 minutes.

3.2.4 Isolation of fungi:

After plating the source materials on MEA, CMA or PDA laced with antibiotics, they were incubated at a temperature of 23-25°C and observed every two days for fungal growth. On emergence of fungal colonies in different zones, aseptic transfer of a portion of the colony was carried out onto fresh MEA/PDA/CMA plates cut into 9 equal sectors. These plates were incubated for 7 days. Growing colonies were compared, observed under stereomicroscope and then transferred to MEA slants (Protocol 1).

3.2.5 Sourcing from culture collection:

Entomopathogenic fungi were isolated indigenously in a joint project of National Institute of Malaria Research, Panaji, Goa with Botany Department, Goa University, Taleigao, Goa, and maintained in the Goa University Fungal Culture Collection (GUFCC) at the Botany Department. Seven of these fungal isolates with known mosquito larvicidal activity obtained for the present study from GUFCC were:

1. *Gliocladium* sp. isolate GUFCC 5039
2. *Gliocladium* sp. isolate GUFCC 5040
3. *Gliocladium* sp. isolate GUFCC 5044
4. *Penicillium* sp. isolate GUFCC 5072
5. *Penicillium* sp. isolate GUFCC 5077
6. *Trichoderma* sp. isolate GUFCC 5088
7. *Trichoderma* sp. isolate GUFCC 5103

These isolates on screening against 2nd instar larvae of *Cx. quinquefasciatus* had shown 100% larvicidal activities in the time interval of 24-48 h except *Trichoderma* sp. isolate GUFCC 5103 which had shown 65% activity on 120 h exposure of the larvae (Keshava Prasad *et al.*, 2000). The isolates showing activity above 50% were

PROTOCOL 1 ISOLATION OF ENTOMOPATHOGENIC FUNGI

Mosquito larvae collected from field

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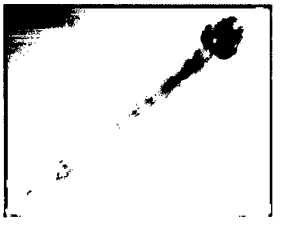
Surface sterilized with 2 % Sodium Hypochlorite solution



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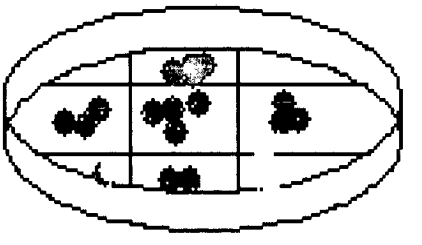
Mixed fungal culture obtained on sector plate



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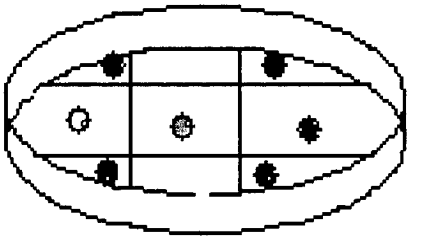
Isolation of fungi on PDA/ MEA



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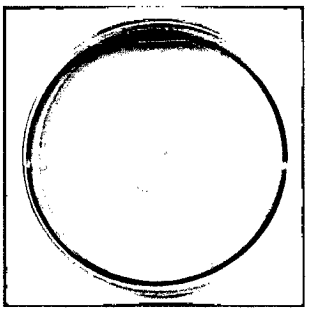
Pure culture of the isolate obtained after 7d



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Pure culture maintained on MEA slants.

considered to have bio-control potential and were considered 'promising isolates'. Hence they were selected for the present study.

From the above isolates four were identified up to species level as follows:

1. Isolate GUFCC 5039 was identified as *Gliocladium roseum* Bain.

2. Isolate GUFCC 5040 was also identified as *Gliocladium roseum* Bain.

3. Isolate GUFCC 5072 was identified as *Penicillium citrinum* Thom

4. Isolate GUFCC 5103 as *Trichoderma atroviride* P. Karsten

The detailed characters of these are discussed in the results chapter.

3.2.6 Maintenance of cultures:

Pure cultures obtained were maintained on malt extract agar slants prepared with 2.5 g dehydrated malt extract and 20 g agar in 1000 ml distilled water (Himedia Laboratories Pvt. Ltd.) without antibiotics in screw capped bottles each containing about 4 ml of medium. The isolates obtained were routinely sub cultured once in every three months.

3.3. Documentation of Fungi:

Source of substrate and the site of collection of the samples were recorded. Based on the cultures established, each species was described based on colony and microscopic characters.

3.3.1 Colony characters:

Each fungal colony was considered as a 'morphotype' and as a species isolate. If the isolate sporulated, it was identified on the basis of the sporulating structures. Along with morphological features, cultural characters of each isolate were recorded. Using standard diagnostic format, descriptions of fungi were written.

3.3.2 Microscopic observations:

Semi-permanent slides of fungi from colonies with sporulating structures such as sporangiophores and sporangia (Zygomycetes) and conidiophores and conidia (Hyphomycetes) were made using lactophenol and cotton blue as mountant and stain and then the slide was sealed with DPX. The slides were numbered and maintained in the Mycolab of Botany Department, Goa University.

Photomicrographs of the isolated fungi on the slides were taken using an automatic camera (Olympus BX41) fitted to the microscope. On the other hand, exogenous fungi growing directly on hosts were photographed using an Olympus Stylus 410 digital camera mounted either onto the Zeiss Stemi 1000 stereoscope or the Wild Heerbrugg M3B binocular stereo microscope.

3.3.3 Identification:

Using standard taxonomic keys, monographs, atlases and websites (Carmichael *et al.*, 1980; Domsch *et al.*, 1980; Samson *et al.*, 1988; Tzean *et al.*, 1997; Gams & Bissett, 1998; The Fungal website, MycoBank: URL: http://www.mycobank.org), various isolates were characterized, identified and assigned to respective genera and species.

3.3.4 Ex situ maintenance and preservation of fungi:

In the collection of 'Goa University Fungus Culture Collection' (GUFCC) a representative pure culture of each identified species or morphotype was maintained.

3.4 Bioassays of mosquito-pathogenic fungi vs. vector mosquito larvae:

3.4.1 Harvesting of conidia and preparation of conidial suspension of test fungal isolates:

a. Corn Meal Agar (CMA) medium as in **3.2.3 a**

b. Potato Dextrose Agar (PDA) medium as in **3.2.3 c**

Each test fungal isolate was grown in plates containing 1.7% CMA or 4.1% PDA at 22-25°C for 14 days. The culture plates were flooded with sterile 0.05% Tween 80 and the spores were harvested by agitating gently with a glass rod or a sterile brush. Tween 80 acts as a surfactant in case of hydrophobic spores thus helping to obtain a homogenous aqueous conidial suspension. The suspension was centrifuged at 6000 rpm for 10 min forming a pellet which was washed in sterile distilled water, re-suspended in minimum amount of water and vortexed. The spore concentration was determined using improved Neubauer's haemocytometer (Goettel & Inglis, 1997). Likewise spore suspension from 21 d culture was also obtained.

3.4.2 Preliminary Bioassays with test fungal isolates:

Preliminary bioassays were carried out to reconfirm pathogenicity of the fungal isolates sourced from GUFCC. The bio-efficacy of each test fungal isolate was assessed against *Cx. quinquefasciatus* 3rd instar larvae using a WHO (1996) recommended standard method. Bioassays were set up in 500 ml plastic bowls with a wide surface area in a final volume of 50 ml sterile distilled water with the dose of 10^5-10^6 spores/ml maintained for all isolates. Five test replicates, each with 20 healthy *Cx. quinquefasciatus* 3rd instar larvae were set up for up to 72 h exposure time depending on the activity previously reported. Three control replicates without the spore suspension were concurrently maintained. The bowls were covered with nylon net secured with a rubber band from out side margin (Fig. 5). Larvae were fed with 20-25 mg baby food Farex ™ daily to prevent starvation. Mortality counts were recorded at 24 h interval based on the number of live larvae remaining in each bowl. Observations on sluggishness or other pathogenic symptoms of the larvae were carried out. Moribund and dead larvae were examined individually under a Wild Heerbrugg M3B binocular stereo microscope. Test isolates affecting more than 50%

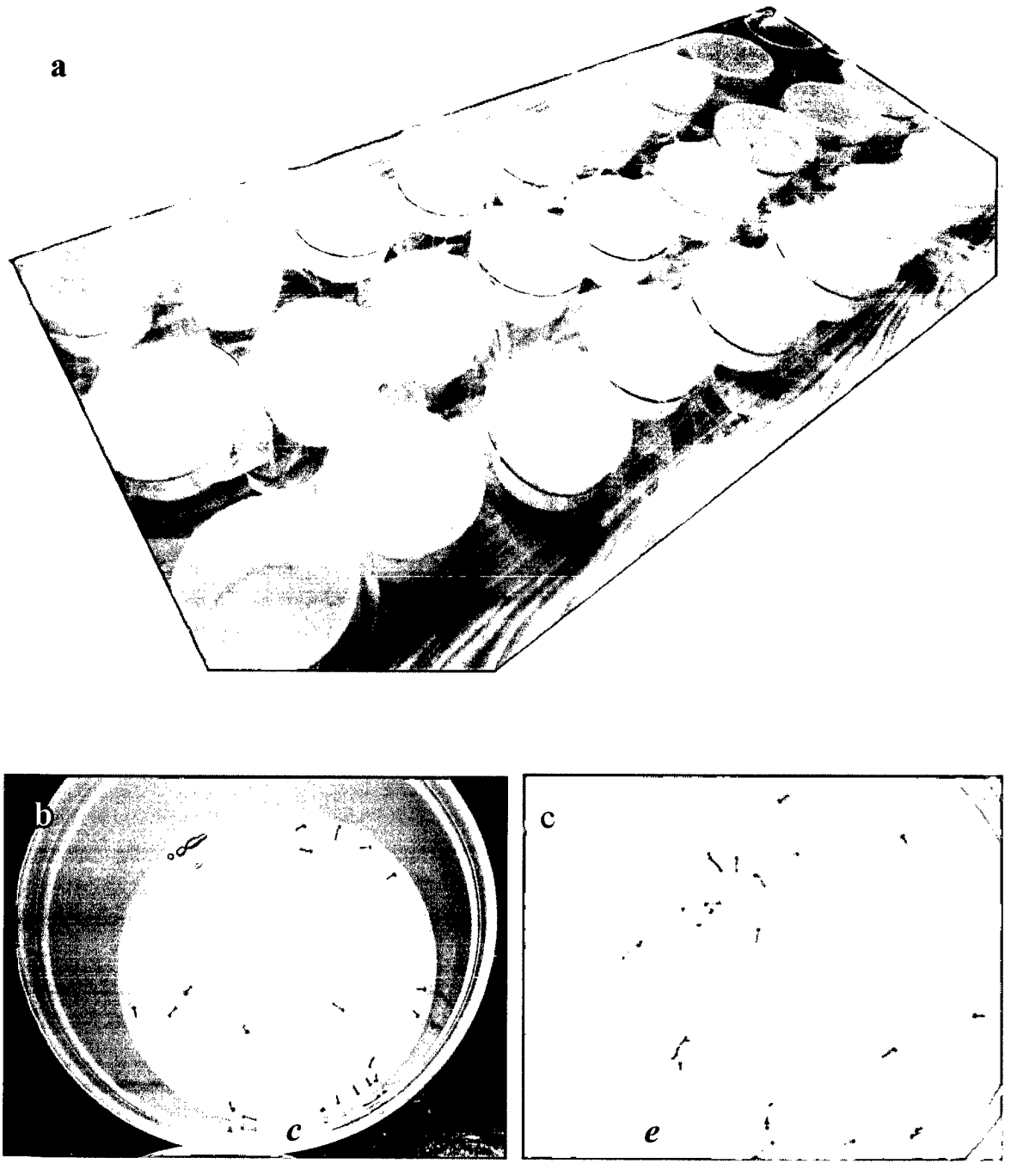


Fig. 5 a. Preliminary bioassay with test fungal isolates against mosquito larvae; **b**. Control larvae **c**. Exposed larvae; *c* = control, *e* = exposed.

mortality were considered as potential biocontrol agents of mosquitoes and referred to as promising isolates.

3.4.3 Corrected mortality:

If larval mortality in control ranged from 5-20%, then 'corrected mortality' was calculated using Abbott's formula (Abbott, 1925) given below.

$$\text{Abbott's Formula For Corrected \% Mortality} = \frac{\text{\% mortality (Test) - \% mortality (Control)}}{\text{100 - \% (mortality Control)}} \times 100$$

Corrected mortality was not necessary whenever the control mortality ranged between 0- 5%, (Plestina, 1984). The bioassay was discarded and experiment was repeated if the control mortality exceeded 20% in order to re-confirm the results obtained.

3.4.4 Mass production of conidia:

In vitro production of conidia of the most promising fungus *P. citrinum* was done in large quantity in culture bottles having a surface area of about 120 cm^2 when kept horizontally. Fifty ml of Malt Czapek Agar medium was poured in each bottle with the medium spread in horizontally placed bottles. Each culture bottle was inoculated with 300 µl of spore suspension @10^5 spores/ml and incubated for 14-16 days. This inoculum was prepared as in 3.4.4.2.

3.4.4.1 Media for mass production of conidia:

Malt Czapek Agar (Solid medium):

Sucrose 30 g; malt extract 40 g; agar 20 g; Czapek (Cz) stock solution A-50 ml; Czapek stock solution C-50 ml; distilled water-900 ml.

Malt extract was dissolved in 900 ml distilled water by boiling; sucrose and 50ml each of Cz stock solution A and C were added, pH was adjusted to 5.5, agar was

added and dissolved. Fifty ml of the medium was dispensed in each culture bottle and sterilized by autoclaving at 15 psi for 15 minutes.

[Cz stock solution A: $NaNO_3$-4 g; KCl-1 g; $MgSO_4.7H_2O$-20 mg; distilled water-100 ml Cz stock solution C: K_2HPO_4-2 g; distilled water- 100 ml]

3.4.4.2 Inoculum preparation:

P. citrinum was grown in petriplates on cornmeal (1.7%) agar medium. On 7th day the spores were harvested in sterile double distilled water with round tipped glass rod and spore suspension was obtained as in **3.4.1**. In horizontally placed culture bottles, in each bottle having a surface area of 120 cm^2, 300 µl of spore suspension @10^5spores/ml was inoculated on nutrient medium and incubated for 14-16 days.

3.4.4.3 Spore suspension preparation: The culture bottles were flooded with 20 ml of sterile 0.05% tween 80 after 14-16 days. Harvesting of spores and spore suspension preparation was as in **3.4.1**.

3.4.5 Main Bioassays:

A dose range for spores was decided based on results of preliminary bioassays carried in 50 ml of water choosing doses arbitrarily so as to obtain 0-100% mortality. Based on the effective doses obtained during preliminary bioassays, main bioassays were conducted in accordance with WHO (1992) main bioassays were set up to study the bio-efficacy of the most promising isolate. For each of the doses tested three replicates were maintained. Twenty five healthy *Cx. quinquefasciatus* 3rd instar larvae were introduced in a final volume of 250 ml water in 500 ml plastic bowls with a surface area of 115 cm^2. The bowls were labeled and kept covered with nylon net to prevent any disturbance. Two negative controls were maintained concurrently with twenty-five larvae each in 250 ml of water without the spore suspension. 20-25 mg Farex ^{TM} was provided as larval feed every 24 hours. Mortality and sluggishness was

observed and recorded at an interval of 24 h for 48 hours. The results were based on the count of live larvae. The larvae which did not show movement when prodded with glass rod were considered dead. Observations were carried out under Wild Heerbrugg M3B to observe the morphological changes and to confirm mortality.

Similar bioassays were carried out using *P. citrinum* 14 d conidial suspension at different doses in 250 ml water against *An. stephensi* 3rd instar larvae and *Ae. aegypti* 3rd instar larvae. The latter was exposed for 72 h due to slow response.

3.4.6 Statistical analysis:

Percentages of larval mortality were determined and corrected mortality data were subjected to two-way Analysis of Variance (ANOVA) using software SPSS version 16. The lethal dose/concentration (LD_{50}/LC_{50}) required to kill 50% of larvae was calculated by Probit analysis (Finney, 1971).

3.5 Mode of action of active fungi in mosquito larvae:

Mode of invasion of three fungal isolates, *Gliocladium* sp. isolate GUFCC 5044, *T. atroviride* and *P. citrinum* was studied in the 3rd instar larvae of test vector species by light microscopy, histological techniques and cryostat sectioning.

3.5.1 Light Microscopy Studies

3.5.1. A Histopathological light microscopic study of mode of action of *Gliocladium* sp. isolate GUFCC 5044:

The mode of action of *Gliocladium* sp. isolate GUFCC 5044 against *Cx. quinquefasciatus* 3rd instar larvae was studied by exposing the larvae to an active dose of 2.7 x 10^6 spores /ml. Three experimental replicates and two control replicates with twenty larvae in each were maintained. The treated larvae were then processed further. The exposed larvae after 24 h exposure and 48 h exposure were fixed in

Altmann's fixative (Gatenby & Beams, 1950) for 2 h and those fixed with Bouins fixative for 24 h to study histopathology. Similarly control larvae were also fixed in both the fixatives. Then they were washed under tap water for 5-10 minutes, subjected to alcohol series i.e. 30%-70% ethanol for 10 minutes in each of the concentrations; 90% and 100% ethanol, for 20 minutes each. Then it was kept in a mixture of absolute ethanol + benzene (1:1) for 5 min followed by treatment with pure benzene for 5 min, Benzene + wax, (1:1) for 30 minutes and two changes of pure wax 30 min each. Paper blocks of rectangular shape 3.5 cm x 7 cm were used as moulds. Embedding was done using paraffin (58-60°C m. p). For microtome sectioning, larvae fixed with both the fixatives were used. On the rotary microtome 6 – 8 µm thick sections were cut.

After spreading the tissue ribbon the slides were dried and then the slides were deparaffinized, down graded in a descending series of alcohol and stained with aqueous haematoxylin. Then upgraded in an ascending series of ethanol and stained with alcoholic eosin. Cleared in benzene/xylene and mounted in D.P.X. / Canada balsam.

Also hourly examination up to 8 h of the treated and control larvae was done under Wild Heerbrugg M3B (16X and 40X) Microscope.

3.5.1. B Light Microscopic study of mode of action of *T. atroviride* by cryostat sectioning:

The position of the conidia of *T. atroviride* in the gut of *Cx. quinquefasciatus* after ingestion was studied by light microscopy. *Cx. quinquefasciatus* 3rd instar larvae were exposed to an active dose of 6.9 x 10^6 spores/ml. After 48 h exposure, the treated and control larvae were fixed in Altmann's fixative for 2 h and sectioned in a Leica M-800 cryostat at –14°C to obtain 20 µm thick longitudinal sections. They were air

dried, stained with Lactophenol Cotton Blue, mounted in DPX and observed under binocular microscope.

3.5.2 Fate of fungal spores:

The viability of conidia of *T. atroviride* isolate in the gut of *Cx. quinquefasciatus* 3rd instar larvae following their ingestion and excretion by the larvae was studied according to Goettel (1988b) with some modifications. Three replicates with 10 *Cx. quinquefasciatus* 3rd instar larvae per replicate were used. Control larvae were maintained in two replicates in water. Two hours prior to the experiment 20-25 mg Farex ™ larval feed was given. Glutting of the larvae with ink was done by placing them in 50 ml sterile suspension of India ink (Himedia) (3 drops ink in 100 ml water) for 24 h at 25°C. After 24 h, in 250 ml bowls the above ink glutted larvae were exposed to 50 ml conidial suspension with concentration of 6.9 x 10^6 spores /ml for 1 h 15 minutes. They were then transferred to fresh 10 ml India ink suspension in 20 ml test tube for 1 hour. The ink suspension and the excreted conidia were centrifuged @ 2000 rpm for 10 minutes. The pellet was resuspended in 1 ml supernatant on a vortex for 2 minutes. Then 0.1 ml of conidial suspension at concentration of 1 x 10^5 conidia/ml was spread on CMA Petri plate supplemented with antibiotic mixture of 60 µg/ml penicillin and 30 µg/ml streptomycin and incubated at 25°C for 24 hours. Then the conidial viability was studied.

3.5.3 Scanning Electron Microscopy studies:

Scanning Electron Microscopic (SEM) studies of mode of action of *P. citrinum*:

To study the mode of action of *P. citrinum* isolate vs. *Cx. quinquefasciatus* and *Ae. aegypti* 3rd instar larvae SEM studies were carried out at National Centre for Antarctic and Ocean Research Vasco-da-Gama, Goa.

Twenty 3rd instar larvae of *Cx. quinquefasciatus* were introduced in a 500 ml bowl containing 50 ml spore suspension of *P. citrinum* isolate @ 10 x 10^6 spores/ml. Similarly *Ae. aegypti* 3rd instar larvae were exposed to the spore suspension @ 80 x 10^6 spores/ml. After 24 h and 48 h the dead larvae were processed further for SEM studies. The larvae were fixed in Altmann's Fixative. The specimens were then dehydrated through t- butyl alcohol series. The specimens were mounted on a stub coated with carbon tape and sputter coated with platinum on a JEOL JFC -1600 Autofine Coater for 30 sec. Then they were mounted on the stage of JEOL JSM-6360LV Scanning Electron Microscope and scanned at various magnifications using 2-18 kV (accelerating voltage). Generally voltages of 3 kV and 5 kV were used. Also micrographs of control larvae and unexposed spores were clicked. The contents of digestive system for presence of spores and changes in the spore architecture if any were observed after processing and dissecting the exposed *Culex* larva.

3.6 Bioassays of secondary metabolites of effective fungi vs. test mosquito larvae:

3.6.1 Media for production of secondary metabolites:

Submerged liquid medium was used to study the production of secondary metabolites.

3.6.1 A. Glucose Peptone Yeast Medium (GPYM): Glucose 10 g, Peptone10 g, Yeast extract 4 g, Soluble starch 10 g were dissolved in distilled water 1000 ml, pH adjusted to 5.5; 100 ml media was dispensed in two 250 ml conical flasks and sterilized in an autoclave at 121°C for 15 minutes.

3.6.1.B. Sabouraud Dextrose Broth (SDB): Sabouraud dextrose broth powder (MO33, Himedia Laboratories Pvt. Ltd.) containing special peptone 10 g L^{-1}; dextrose 20 g $L^{-1}$and pH 5.6$\pm$0.2 was dissolved in distilled water (30 g L^{-1}).

3.6.2 Preparation of Cell Free Extract (CFE) by sonication:

The flasks containing sterilized GPYM were inoculated with conidia of *T. atroviride* isolate from 14 d old CMA culture and incubated at 25.8 ± 2°C for 10 days at 150 rpm on an Orbitek rotary shaker. The buff coloured liquid culture obtained was sonicated using electronic sonicator for 2 min yielding a homogenous brown coloured liquid. The liquid was centrifuged for 20 min at 5500 rpm, the residual pellet discarded and the CFE used for bioassay. An uninoculated culture medium served as control.

3.6.3 Bioassays with metabolites of *T. atroviride*:

Screening assay with crude metabolites from the CFE of *T. atroviride* isolate against *Cx. quinquefasciatus* larvae was carried with 24 h exposure time. Based on these results main assays were set up with three different concentrations of metabolites 1µl/ml, 10µl/ml and 100µl/ml. Five replicates of 10 *Cx. quinquefasciatus* larvae per replicate per dose were set in 500 ml plastic bowls with total volume made to 50 ml with deionised water. Concurrently three controls with respective concentration of media were maintained. In each bowl 20-25 mg baby food Farex ™ was provided as food. The bowls were covered with nylon net secured with a rubber band. Larval mortality was recorded at 24 h and 48 hours.

3.6.4 Extraction of secondary metabolites using organic solvents:

Fifty ml of the SDB was dispensed in 250 ml conical flasks and autoclaved at 15 psi for 15 minutes. Agar plugs of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 grown on MEA Petri plates were inoculated in conical flasks with an inoculation needle. The culture flasks were incubated at 24 ± 2° C. Three flasks were picked at the end of 14th and 20th day and processed further.

Preparation of extracellular metabolite extract

According to Mohanty and Prakash (2004), the extracellular metabolites (crude extract) of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 were obtained by filtering the broth through Whatman No. 1 filter paper. The cell free filtrates were then screened for larvicidal activity against 3rd instar larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti*.

Preparation of intracellular metabolite extract

Modified method of Devi *et al.* (1997) was followed and the broth of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 was filtered through Whatman No. 1 filter paper. The biomass filter cake/mycelial mat was collected and weighed. The wet weight was noted. It was washed with distilled water twice, ground in a mortar and pestle to a fine paste and then extracted with 25 ml 100% methanol twice in a conical flask with vigorous shaking. Each extraction lasted for one hour. The extracts were then combined, frozen and lyophilized and concentrated. The cell free filtrates were then screened for larvicidal activity.

3.6.5 Screening of fungal secondary metabolites for larvicidal activity:

Larvicidal bioassay of crude extract

Cell free culture filtrates containing extracellular metabolites of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 were tested against *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* 3rd instar larvae for larvicidal activity. Twenty larvae were placed in 250 ml plastic bowls containing 10 ml of crude metabolite extract and 40 ml tap water. Three replicates were maintained. Two controls were maintained with 10 ml of culture medium and 40 ml tap water. 20-25 mg farex was provided as larval feed every 24 h. Readings were noted after 24 h and 48 hours.

Larvicidal bioassay of methanol crude extract

Larvicidal activities of methanol crude extract containing intracellular metabolites of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 (both isolates incubated for 14 days) were tested against *Ae. aegypti* and *An. stephensi* 3rd instar larvae. *Penicillium* sp. isolate GUFCC 5077 was also tested against *Culex quinquefasciatus* 3rd instar larvae. Methanol crude extract of 20 days culture of *Penicillium* sp. isolate GUFCC 5072 was also tested against *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* 3rd instar larvae.

Twenty larvae were placed in 250 ml plastic bowls containing 50 µl of test fungal methanol extract and 49950 µl tap water. Three replicates were maintained. Two controls were maintained with 50 µl of methanol and 49950 µl tap water. 20-25 mg Farex™ was provided as larval feed every 24 h. Readings were noted after 24 h and 48 hours.

3.7 Partial Purification of effective secondary metabolites:

3.7.1 Media for mass production of secondary metabolites:

Submerged liquid medium of SDB was used for mass production of secondary metabolites of *P. citrinum* isolate.

SDB as in 3.6.1.B.

Fifty ml of SDB media per bottle was dispensed in culture bottles each having a surface area of 120 cm^2. The bottles were autoclaved at 15 psi for 15 min. 100 µl of spore suspension of *P. citrinum* isolate @10^5 spores/ml grown on 5% MEA slants for 14 days was inoculated in each culture bottle. The bottles were incubated at 24 $\pm$ 2°C. All the bottles were picked at the end of 14th day and processed further (Fig. 6 a).

Preparation of intracellular metabolite methanol extract

Modified method of Devi *et al.*, (1997) was followed. The *P. citrinum* isolate GUFCC 5072 broth was filtered through Whatman No. 1 filter paper. The biomass filter cake/mycelial mat was collected and weighed. The wet weight was noted. It was immersed in 125ml methanol in a 250 ml flask plugged with cotton for 60 h. The mat was removed from methanol. The methanol extract was collected in a conical flask. The mat was then ground in blender to a fine paste and immersed in 100% methanol in a conical flask with vigorous shaking. Each extraction lasted for half an hour then it was filtered and the residue was immersed in methanol again. This was repeated five times. The extracts were then combined, frozen and lyophilized and the concentrated extract refrigerated until further use.

3.7.2 Separation of crude metabolites:

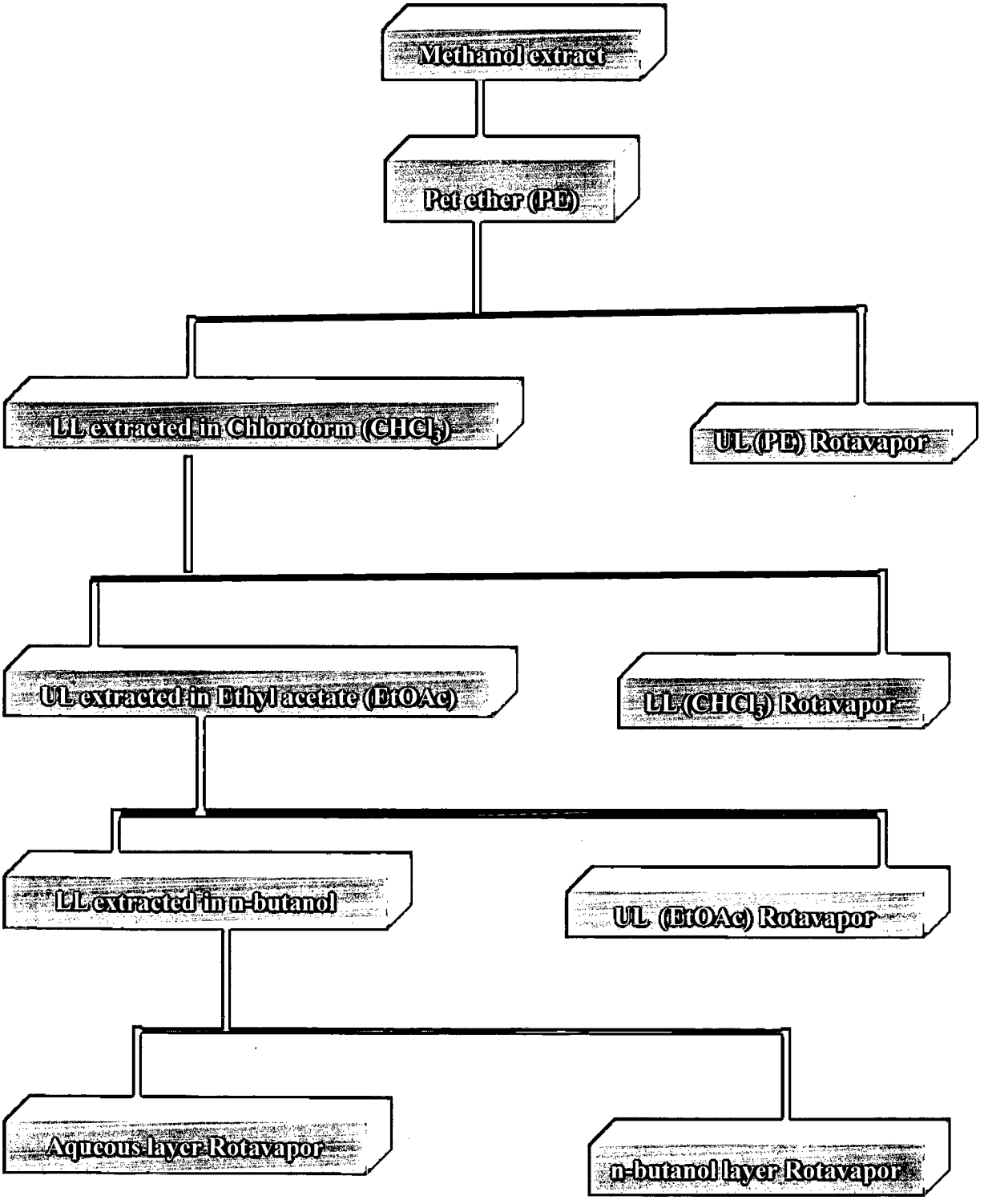
Separation and partial purification of the concentrated methanol extract of *P. citrinum* isolate was achieved as shown in Protocol 2 in the following manner (Fig. 6 b-e):

The concentrated methanol extract was extracted in Pet ether thrice in a separating funnel. The pooled Pet ether fraction was then reduced to yellow granular concentrate in a rotavapor. The remaining lower aqueous layer was then extracted in chloroform thrice and concentrated in a rotavapor. The upper aqueous layer was then extracted in Ethyl acetate thrice and reduced in a rotavapor. Now the lower layer was subjected to n-Butanol extraction thrice and evaporated in rotavapor. The aqueous layer remaining was then lyophilized and concentrated. Weight of each fraction was calculated. Bioassays were set up to check the bioactivity of each fraction. Each fraction was re-dissolved in either Dimethyl sulphoxide (DMSO) or methanol to run the bioassays.

3.7.3 Bioassays of Partially Purified fractions vs. mosquito larva:

Bioassays of Partially Purified Fractions of *P. citrinum* isolate were run against *Cx.*

PROTOCOL 2 FOR SEPARATION OF METABOLITES



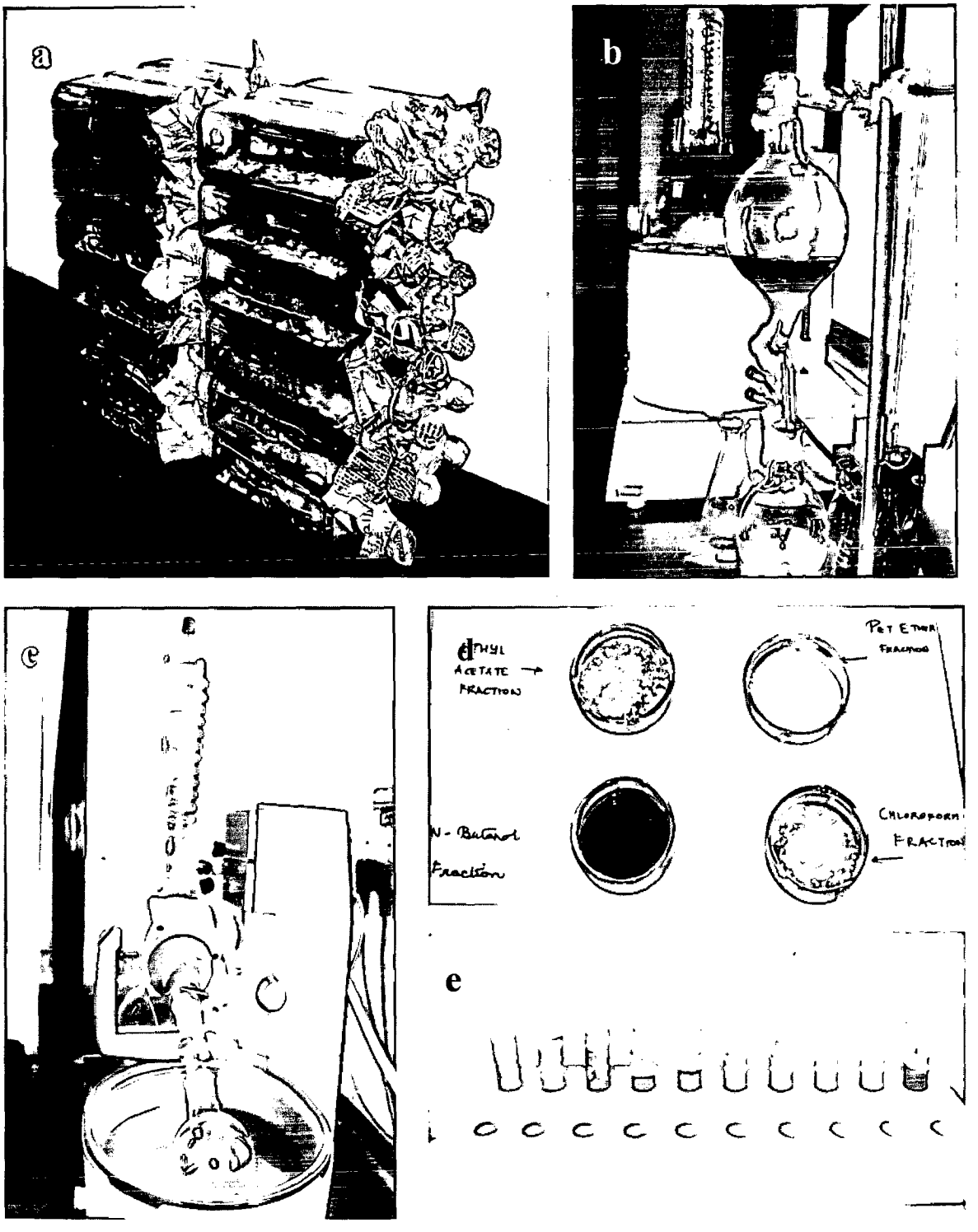


Fig. 6 a. *In vitro* mass culturing of *Penicillium citrinum* on Sabouraud Dextrose Broth in bottles **b.** Separation of fractions **c.** Concentrating the fractions in rotovapor **d.** Dried fractions **e.** Each dried fraction redissolved in appropriate solvent to test activity.

quinquefasciatus 3rd instar larvae (Fig. 7). For each of the separated fractions bioassays were set up with two control and three experimental replicates in a final volume of 50000 µl aerated tap water. The bioassays were set in 500 ml plastic bowls with 500 µl of the respective fraction in the experimental bowls whereas in the control bowls 500 µl of methanol or DMSO or water was used depending on the solvent used to dissolve the fraction. In each replicate twenty *Cx. quinquefasciatus* 3rd instar larvae were maintained. All the bowls were covered with nylon net. The assays were run for 48 hours. Readings were noted every 24 h and % mortalities were calculated.

3.7.4 Thin layer Chromatography of active fractions:

To separate compounds based upon their polarity and interaction with silica and to assess their purity the Thin Layer Chromatography of methanol, pet ether and chloroform fractions was carried out using two solvent fronts:

1) 30% ethyl acetate in Pet Ether.

2) 25% ethyl acetate in Pet Ether.

The plates were subjected to iodine chamber. Retardation factor (Rf) values were calculated.

3.7.5 Nuclear Magnetic Resonance of active fraction:

Nuclear magnetic resonance (NMR) spectra of Pet Ether fraction was obtained at NIO. ^{1}H nuclear magnetic resonance (NMR) spectra was obtained on Bruker model DRX 400 avance spectrometer with standard pulse sequences operating at 300 MHz for ^{1}H NMR with deutero chloroform ($CDCl_3$) as the solvent and TMS as the internal standard. Looking at the NMR spectral pattern the Pet Ether fraction was processed further. Chemical separation of recovered NMR fraction was carried out in the Chemistry Dept, Goa University. To the Pet ether fraction 1 ml of diethyl ether was added and transferred to a separating funnel. To this, 4 ml diethyl ether and 5 ml

saturated sodium bicarbonate were added and mixed gently. Now the upper yellow diethyl ether layer was transferred to a conical flask and anhydrous sodium sulphate (Loba chemie) was added to remove excess moisture. This was transferred to a test tube (t. t) and referred to as t. t 1. The lower sodium bicarbonate layer was then acidified with conc. HCl. Then it was transferred to a separating funnel and diethyl ether was added again to extract any acids if present. The lower aqueous layer was decanted and the upper layer was taken in a t. t., to this anhydrous sodium sulphate (Loba chemie) was added and kept in a water bath for 3-4 min to evaporate. This test tube was referred to as t. t 2 and acids if any should be seen in the spectra of this sample. Second NMR of the two samples obtained above was then taken.

3.7.6 Infra red Spectroscopy of active fraction:

The two compounds were labeled as DR1 Org. layer and DR 2 $NaHCO_3$. They were subjected to Fourier Transformed Infrared Spectroscopy (FT-IR) at National Institute of Oceanography (CSIR), Donapaula, Goa. Infrared spectra were recorded on a SHIMADZU FTIR Prestige-21 spectrometer. The samples were analyzed as KBr pellets.

3.8 Enzyme assays of active fungal isolates:

3.8.1 Protease activity assay:

3.8.1 A Media for Protease activity assay:

Czapek Dox broth (CzDB) media

Czapek Dox broth (CzDB) medium consisted of Glucose-10 g; Sodium nitrate 3 g; Dipotassium Hydrogen Phosphate-1 g; Magnesium sulphate- 0.5 g; Potassium chloride- 0.5 g; Ferrous sulphate- 0.01 g. All the components were dissolved in 1000 ml distilled and pH was adjusted to 7.3.

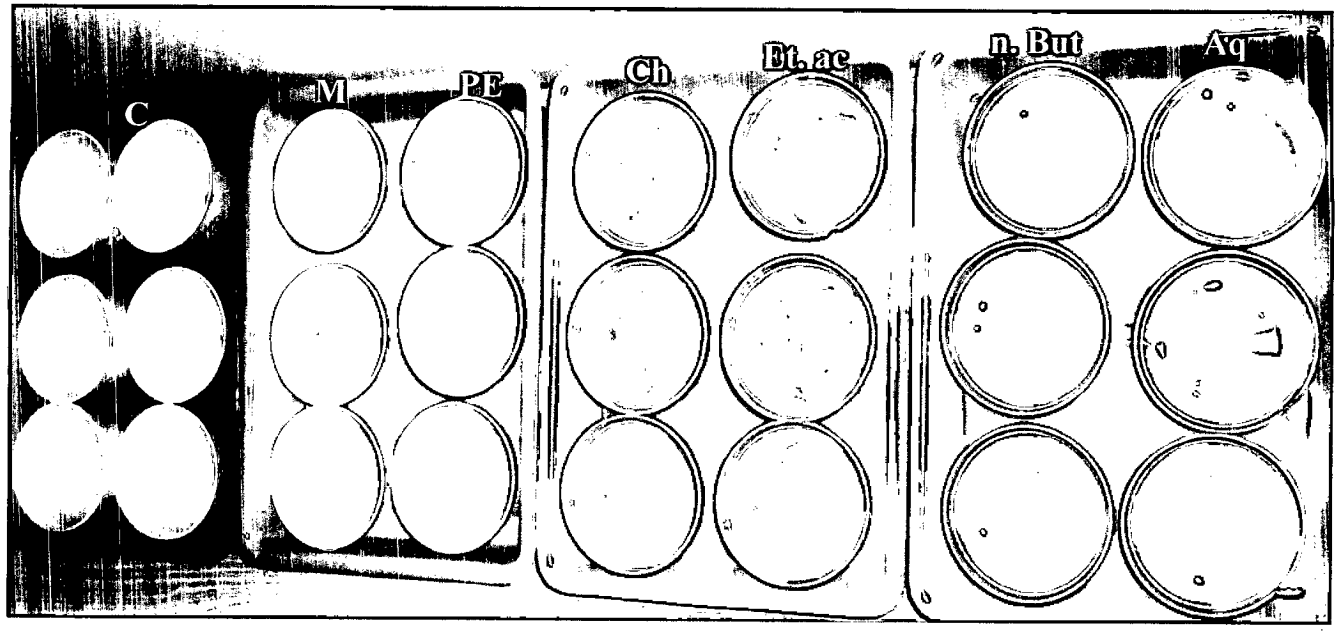


Fig. 7 Bioassay of partially purified fractions of *P. citrinum* vs. *Cx. quinquefasciatus* 3rd instar larvae; C = Control, M = Methanol, PE = Pet ether, Ch = Chloroform, Et. Ac = Ethyl acetate, n.But = n-Butanol, Aq.= Aqueous.

Fig. 8 Culture of *G. roseum* (isolate GUFCC 5039) and *P. citrinum* on Czapek Dox broth for Protease assay.

Before adjusting the pH, 0.25% of skimmed milk was added to the media.

3.8.1 B Protease activity assay:

P. citrinum isolate grown for 14 d on CzDB media with 0.25% skimmed milk (pH 7.3) was assayed for protease activity. At the end of 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 7^{th}, and 14^{th} day the broth flasks were picked and under aseptic conditions the fungal culture was filtered using Whatman no. 41 filter paper to yield 2 ml of enzyme plus broth mixture. Seven days culture of *G. roseum* isolate GUFCC 5039 grown on CzDB media with 0.25% skimmed milk (pH 7.3) was also assayed for protease activity (Fig. 8).

Protease activity was measured by a modification of Calik *et al.* (1998) method. The following procedure was used for assaying the protease activity:

A series of Tyrosine concentration gradient ranging from 10 to 100 µg /ml and from 100 to 1000 µg/ml were prepared using 1.1 mM L-Tyrosine standard stock solution (0.2 mg/ml). To each concentration 1 ml of 2-fold diluted Folin-Ciocalteau reagent, 2 ml 20 % sodium bicarbonate and 5 ml distilled water was added. The optical density of the mixture was read at 660 nm on spectrophotometer (Shimadzu UV-2450 spectrophotometer). A standard graph of concentration in µg /ml v/s absorbance at 660 nm was plotted. The test fungal culture filtrate was assayed for protease activity. Exactly 2 ml culture filtrate was incubated with 2 ml of 1% casein solution for 45 min at room temperature. To stop the reaction 2 ml 5% Trichloroacetic acid (TCA) was added and incubated further for 45 min at room temp. The precipitated protein was pelleted down by centrifuging for 15 min at 6000 rpm. About 1 ml supernatant was assayed for liberated tyrosine after adding 1ml of 2-fold diluted Folin-Ciocalteau reagent, 2 ml 20% sodium bicarbonate and 5 ml distilled water. O.D readings were taken at 660nm on Shimadzu UV-2450 spectrophotometer. Media blank containing filtrate was also read. The amount of tyrosine liberated was estimated by MS excel

program from standard calibration curve of tyrosine. One unit of protease is defined as the amount of enzyme which liberates 1µg of tyrosine min^{-1} ml^{-1} under standard assay conditions.

3.8.2 Chitinase activity assay:

3.8.2. A. Media for chitinase assay:

Composition of Chitin Broth Media (per 1000 ml)

K_2HPO_4–1.0 gm; KH_2PO_4– 3.0 gm; $MgSO_4$ 0.7 gm; $(NH4)_2SO_4$ -- 1.4 gm;

NaCl --0.5 gm; $CaCl_2$ 0.5 gm; Yeast Ext. 0.5 gm; Bacto-Peptone 0.5 gm;

Chitin 5.0 gm and Distilled water 1000 ml.

P. citrinum isolate culture was grown on PDA for 5 days. From sporulating culture, 1 cm agar piece was cut and inoculated in the 100 ml Chitin broth containing 0.5% chitin powder (Sigma). Flasks were incubated at room temperature under shaking conditions (200 rpm). Samples were taken out at every 24 h time intervals after inoculation up to 96 hours.

3.8.2. b. Chitinase activity assay:

Chitinase activity (U ml^{-1} min^{-1}) of *P. citrinum* isolate with respect to incubation time was assayed in the manner given below.

To 1 ml culture filtrate (enzyme) was added 1 ml of acetate buffer (0.05M, pH 5.0) and 1 ml of acid swollen chitin (substrate). In the control tubes inactivated enzymes were added. The tubes were incubated at 50°C for 1 hour. Using Morgan and Elson (1934) Method 0.5 ml of supernatant was estimated for N-acetyl ß-D glucosamine (NAG).

To 0.5 ml of sample 100 µl Borate buffer (pH 9.2, 0.02M) was added and kept in boiling water bath for 3 min. Cooled under running tap water. To this 3 ml of p-

dimethyl Amino Benzaldehyde (DMAB) reagent was added and incubated at 37°C for 20 min after mixing. At 585 nm the O.D was taken on a spectrophotometer.

3.9 Bio-safety of the effective fungus to non-target organisms:

3.9.1 Field Collection and acclimatization of the vertebrate non-target organism:

To test safety of the efficacious fungus to non-target organisms (NTO) a fish belonging to *Aplocheilus sp.* was used as a vertebrate model. This fish is a larvivorous fish which preys on the mosquito larvae. It was procured from the back waters of Taleigao, Panjim, Goa. To acclimatize them in the laboratory they were then introduced and maintained in the lab conditions in aquaria filled with ten liters aerated tap water for a week. Hitachi fish feed was given everyday.

3.9.2 Bioassay of effective fungus vs. *Aplocheilus blocki*:

To study the biosafety of *P. citrinum* isolate to the NTO *Aplocheilus blocki* a bioassay was set up at NIMR microbiology lab in plastic bowls each with a surface area of 572.78 cm^2 and a height of 11 cm (Fig. 9 A). One control and two experimental replicates were used. Each bowl was aerated with the help of an aerator pump which was attached to a 'T' junction; from this divider three rubber tubes were attached. A bubble controller was introduced in each of the tubes and at the opposite end a diffuser stone. The water was aerated for 24 h. In the control bowl 2000 ml aerated tap water was used (Fig. 9 B). In the 2 experimental bowls 40 ml of *P. citrinum* sp. isolate spore suspension was introduced i.e. 2% of total volume in each the resultant was having a concentration of 34.76 $x10^7$ spores /ml (Fig. 9 C). This was allowed to mix with the water for 30 min. Then in each of the bowls 6 fishes were introduced. Same number of fishes were introduced in the control bowl which was without the spore suspension. All the bowls were covered with nylon net. Hitachi fish feed was

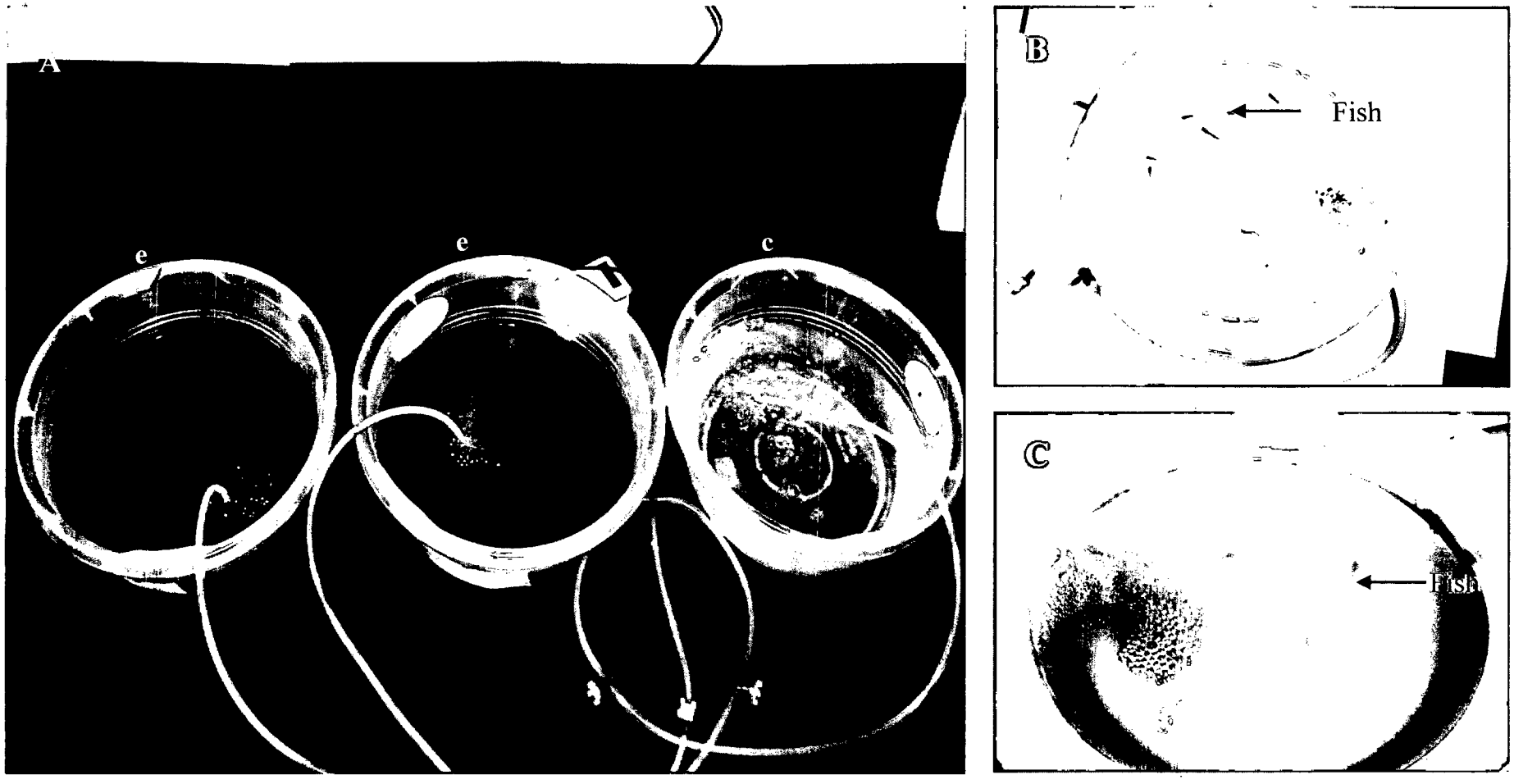


Fig. 9. A. Bioassay of *Penicillium citrinum* against vertebrate non-target organism *Aplocheilus blocki* (Larvivorous); **c**=control, **e**= exposed. **B**. Control **C**. Experimental

given every day. The fishes were observed for morphological changes and readings were taken to see the mortality/survival for seven days.

3.9.3 Field collection and acclimatization of the invertebrate non-target organism:

To test the bio-safety of the efficacious fungus to invertebrate NTO semi aquatic heteropteran bugs collected from a local pond were used. This water strider was chosen as it shared the habitat with mosquito larvae. After scouting eight areas in Calangute and three in Panjim, the bugs were located in sufficient numbers at Panjim. These insects used for the study were collected from a pond situated at Campal, Panjim. They were collected with dip nets. They were transferred to 250 ml pet jars covered with nylon nets (Fig. 10 A-C). The acclimatization of the invertebrate non-target Heteropteran insects to the lab conditions was done by introducing and maintaining them in aquaria each filled with ten liters of pond water brought from the same pond. The bugs were given a meal of *Anopheles* and *Culex* larva and maintained for ten days.

3.9.4 Bio-safety assay of spore suspension of effective fungus vs. Heteropteran insect:

To study the effect of spore suspension of *P. citrinum* isolate on the non target organism a bioassay was set up in plastic bowls each with a surface area of 573 cm^2 and a height of 11 cm. Two control and five experimental replicates were used for the bioassay (Fig. 11). Tap water which was aerated for 24 h was used. In each bowl 50 *An. stephensi* 3^{rd} instar larvae were introduced as feed for the bugs. After every 48 h the old batch of larvae were siphoned out and fresh larvae were introduced.

In each control replicate 3000 ml tap water was used. In experimental replicates a

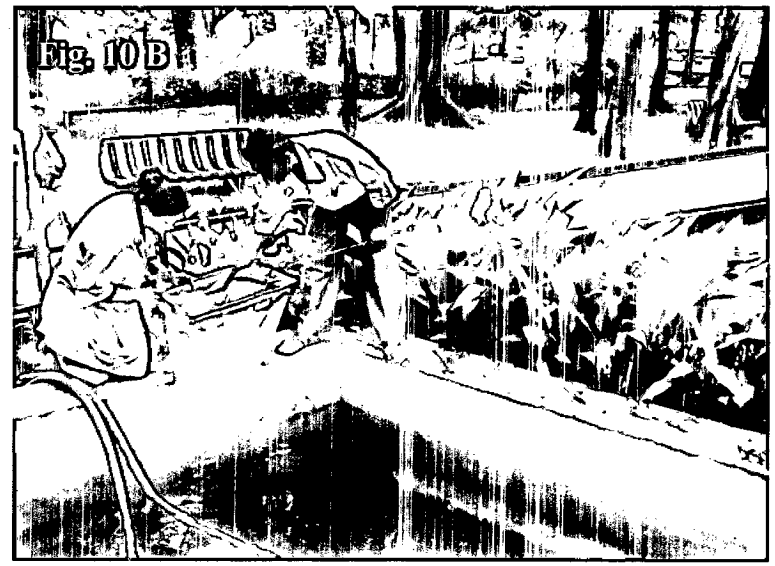


Fig. 10 A-C. Field collection of non-target insect *Limnogonus (Limnogonus) fossarum fossarum*
Fig. 11 Bioassay of *Penicillium citrinum* conidial suspension to study its bio-safety vs. *L. (Limnogonus) fossarum fossarum*; **c**=control, **e**= exposed.

final volume of 3000 ml was obtained by mixing 2918.73 ml tap water with 81.264 ml conidial suspension of *P. citrinum* isolate to give a concentration of 3 x 10^8 spores/ml.

Five heteropteran bugs were transferred in each control and experimental replicate. The bowls were covered with nylon nets. The bugs were exposed for 4 days to the suspension and the observations were carried out for 10 days. The control and exposed bugs were anaesthetized and were examined under Wild M3B Heerbrugg Stereo Microscope.

The parameters studied in the bugs were sluggishness, exogenous growth of the fungus on body surface and mortality. Two days prior to the above bioassay the viability of the *P. citrinum* isolate spore suspension was confirmed by setting up a larvicidal bioassay of the same against *An. stephensi* 3rd instar larvae using LD_{50} value of 17.13 x10^6 spores/ml for 24 hours.

3.9.5 Bio-safety assay of secondary metabolites of effective fungus vs. Heteropteran insect:

The bio-safety of crude metabolites of *P. citrinum* isolate to the non target Heteropteran insect was tested by setting up bioassays. They were set up with two control and three experimental replicates in a final volume of 10 litres pond water in aquaria (Fig. 12). In each aquarium 50 *Cx. quinquefasciatus* 3rd instar larvae were introduced as feed for the bugs. A few weeds were introduced alongwith the pond water. From the heteropteran bugs maintained in the plastic bowls one bug at a time was directly introduced in a test tube. A cotton wad with two drops of ether was introduced in the test tube, after 30 sec the bugs were anaesthetized. After 4-5 min they revived and were active again. Five bugs which were anaesthetized and revived were maintained in each control. In a total volume of 50000 µl having 48005 µl water

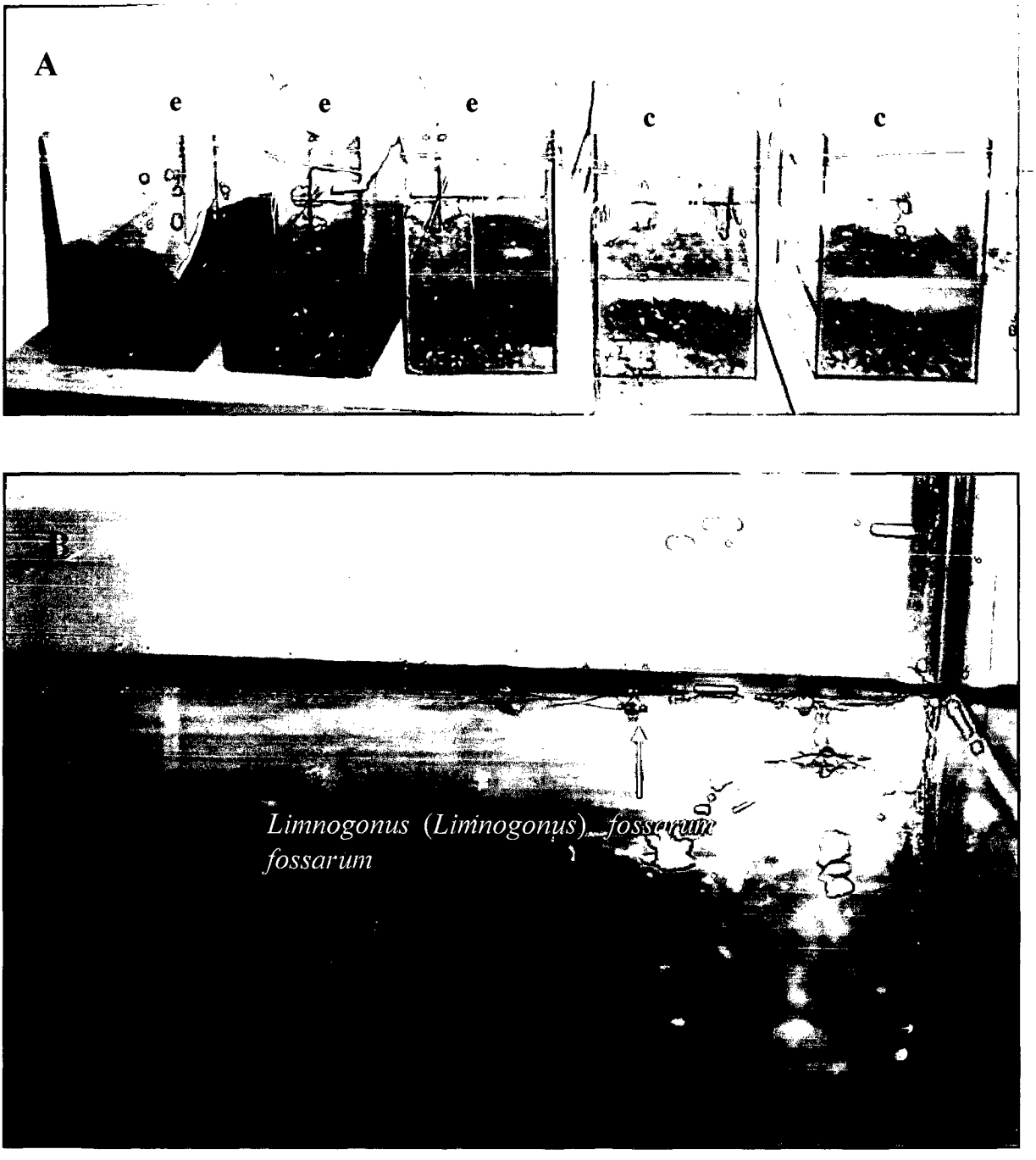


Fig. 12 A. Bioassay of *Penicillium citrinum* crude metabolites (methanolic extract) vs. non-target insect *Limnogonus* (*Limnogonus*) *fossarum fossarum* c = control ; e = exposed B. Magnified view of an aquarium with the exposed insects.

and 1995 µl *P.citrinum* methanol extract containing crude metabolites anaesthetized bugs were dipped for 10 sec from ventral side and exposed to the solution for 10 sec on the dorsal side. The bugs revived after 4-5 min, and then they were introduced in the aquaria and maintained as experimental for 48 hours. The control and experimental aquaria were covered with nylon nets. Observations to study mortality and sluggishness in the non-target heteropteran bugs were carried out and recorded after 24 h and 48 hours.

3.9.6 Identification of the Heteropteran insect:

The heteropteran insect was identified until generic level using Epler's manual (2006). It was further identified until the species level by Dr. Thirumalai from Zoological Survey of India (ZSI), Chennai.

Chapter 4

RESULTS

4.1 Sourcing of entomopathogenic fungi and taxonomy:

4.1.1 Sourcing and isolation of entomopathogenic fungi from nature:

From a moderate collection of samples, ten isolates of fungi were recovered in pure culture (Table 7). Entomogenous fungi were sourced from 6 different localities of Goa. Out of the ten fungal isolates recovered four isolates were obtained by using mosquito larval baits in simulation experiments, two isolates were obtained from *Anopheles* adult and four isolates from insects gathered from plant foliage and litter.

4.1.2 Identification:

All the ten fungal isolates were sporulating. The ten isolates were assigned to 4 genera of fungi. They belonged to Zygomycetes (01) and Hyphomycetes/anamorphic fungi (09). Using standard taxonomic keys and monographs the identification of all the fungi isolated was confirmed upto the generic level and few fungi were further identified upto the species level. Below is the detailed description of the fungi.

ZYGOMYCETES

1. *Mucor* sp. (Fig. 14.b)

Entomogenous zygomycete. Fast growing colony with a diam. of 9 cm in 7 d; white cottony/filamentous mycelia. Reverse side of the colony light yellow, no odour. Aerial mycelia fill the whole petriplate. Sporangiophores 96-160 x 6-8.5 µm, arising singly from the stolons but sometimes up to 3 together, smooth, erect, coenocytic, occasionally with septa in older colonies; rhizoids few, 8-20 µm wide, up to 58 µm long, hyaline, smooth. Sporangia 32-40 µm diam., globose. Columellae 10-25 µm diam., globose to oval, with a collar, smooth, smaller columellae frequently showing several small warty projections at the tip up to 2.5 µm high. Sporangiospores oblong to elliptical, smooth, grey in mass. Chlamydospores not seen.

Table 7: Entomopathogenic fungi isolated from different localities in Goa.

Name of the species	Code	Substrate	Locality
Aspergillus niger	D2	Longhorned beetle (*Neocerambyx raddei*)	Farmagudi
"	D4	*Anopheles* adult	Panaji
Aspergillus sp.1	D3	Longhorned beetle (*Neocerambyx raddei*)	Taleigao Plateau
Aspergillus sp. 2	D8	*Anopheles* adult	Panaji
Aspergillus sp.3	D9	Longhorned beetle (*Neocerambyx raddei*)	Farmagudi
Fusarium oxysporum	D1	*Ae. aegypti* 3rd instar larvae	Carambolim
F. solani	D7	*An. stephensi* 3rd instar larvae	Saat Mancher, Carambolim
Fusarium sp.	D5	*Anopheles stephensi* 3rd instar larvae	Banastarim
Mucor sp.	D10	Longhorned beetle (*Neocerambyx raddei*)	Farmagudi
Penicillium sp.	D6	*An. stephensi* 3rd instar larvae	Saat Mancher, Carambolim

Specimen examined: On a Long Horned Beetle, from Farmagudi, Goa, India, 29°C, 19.11.2004; leg. DP, direct isolation, Culture No.GU/BOT/NIMR/, Slide No. D10.

2. *Aspergillus niger* Tiegh., *Annls Sci. Nat.*, Bot., sér. 5 **8**: 240 (1867) **(Fig. 13h; Fig. 14i, j and k)**

Entomogenous hyphomycete. *Colonies* on PDA circular, flat, carbonaceous to very deep brownish black with white fringed border, with concentric rings of erect conidiophores possessing globose black aspergillia, attaining a diam. of 6.2-6.8 cm in 7 days, reverse of the colony creamish coloured with folds radiating from centre. *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 2-4 µm wide hyphae. *Conidiophores* mononematous, smooth, aseptate, unbranched, thin-walled, hyaline, 400-600 x 5-7.5 µm, with pyriform, 25-50 µm in diam. vesicles. *Conidiogenous cells* phialidic, on biseriate metulae, discrete, bottle-shaped, covering entire vesicle, 4.5-6.5 x 2.5-3.5 µm. *Metulae* cylindrical, smooth, brown, 5-7 x 3-4.5 µm. *Conidia* catenate, globose to subglobose, very rough or echinulate, thick-walled, 1-celled, black, 3-5 µm diameter.

Specimen examined: On a Long Horned Beetle, from Farmagudi, Goa, India, 29°C, 19.11.2004; leg. DP, direct isolation, Culture No.GU/BOT/NIMR/, Slide No.D2. Additional specimen examined: On an infested *Anopheles* adult, from NIMR, Panaji, Goa, India, 24°C, 4.02.2006; leg. DP, direct isolation, Culture No.GU/BOT/NIMR/, Slide No. D4.

3. *Aspergillus* sp. 1 (Fig. 13g; Fig. 14g)

Entomogenous hyphomycete. *Colonies* on PDA circular, green with concentric rings of erect conidiophores possessing columnar yellowish green aspergillia, attaining a diam. of 4-5 cm in 7 days, reverse of the colony creamish with yellow tint. *Mycelium* submerged composed of septate, branched, smooth, thin-walled, hyaline, 2-4 µm wide

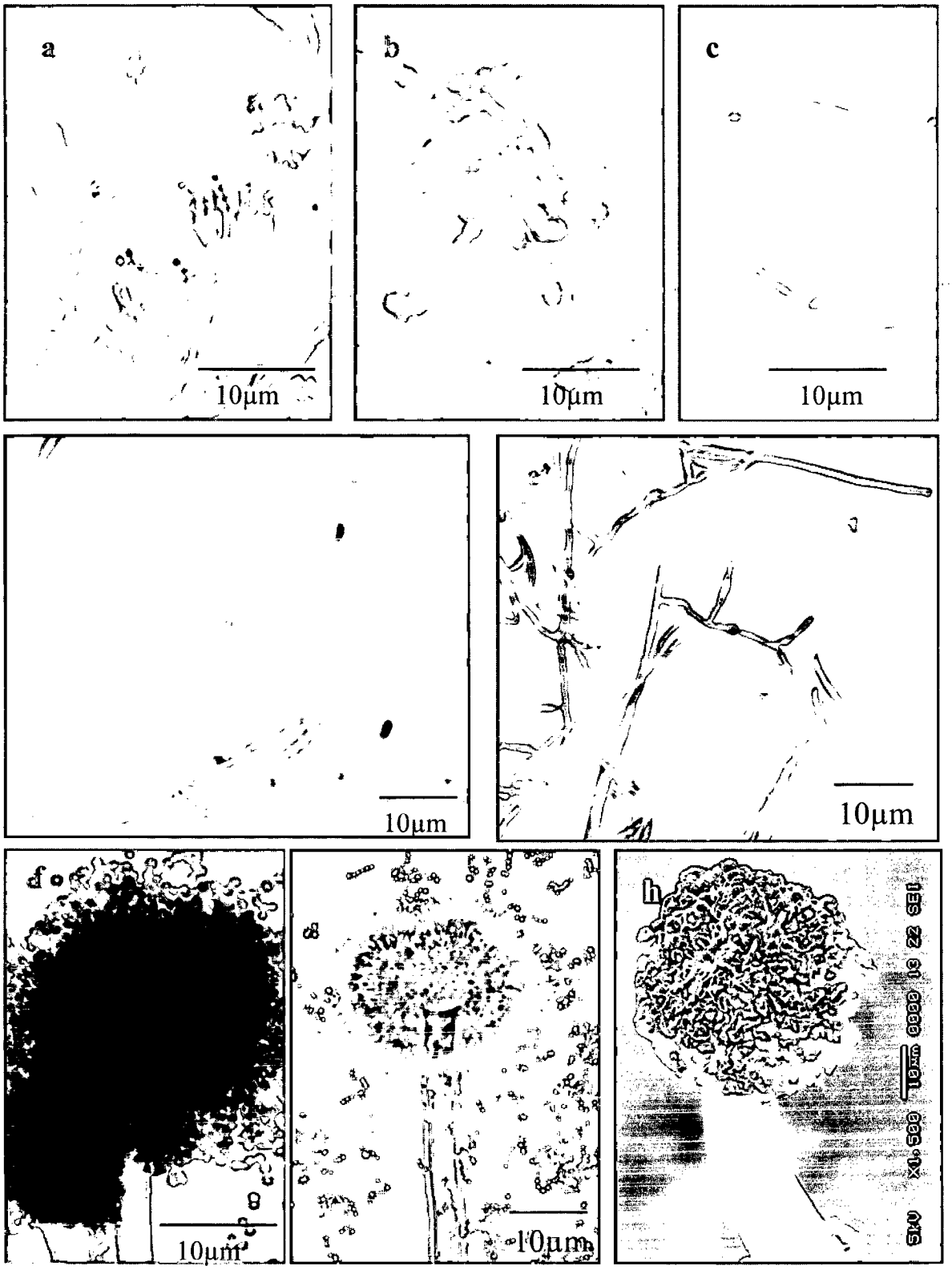


Fig. 13 Microscopic characters of Fungi sourced from mosquito and other insects a. *Penicillium* sp., 100X; b-d. *Fusarium solani* b. Chlamydospores, 100X; c. Macroconidia, 100X; d. Conidiophores, 40X; (Phase contrast) e. *Fusarium oxysporum,* 40X; f. *Aspergillus* sp. 3, 100X; g. *Aspergillus* sp.1, 40X; (Phase contrast) h. *Aspergillus niger* isolate D4, conidiophore 1500X; (Scanning Electron Micrograph) Scale bar =10µm.

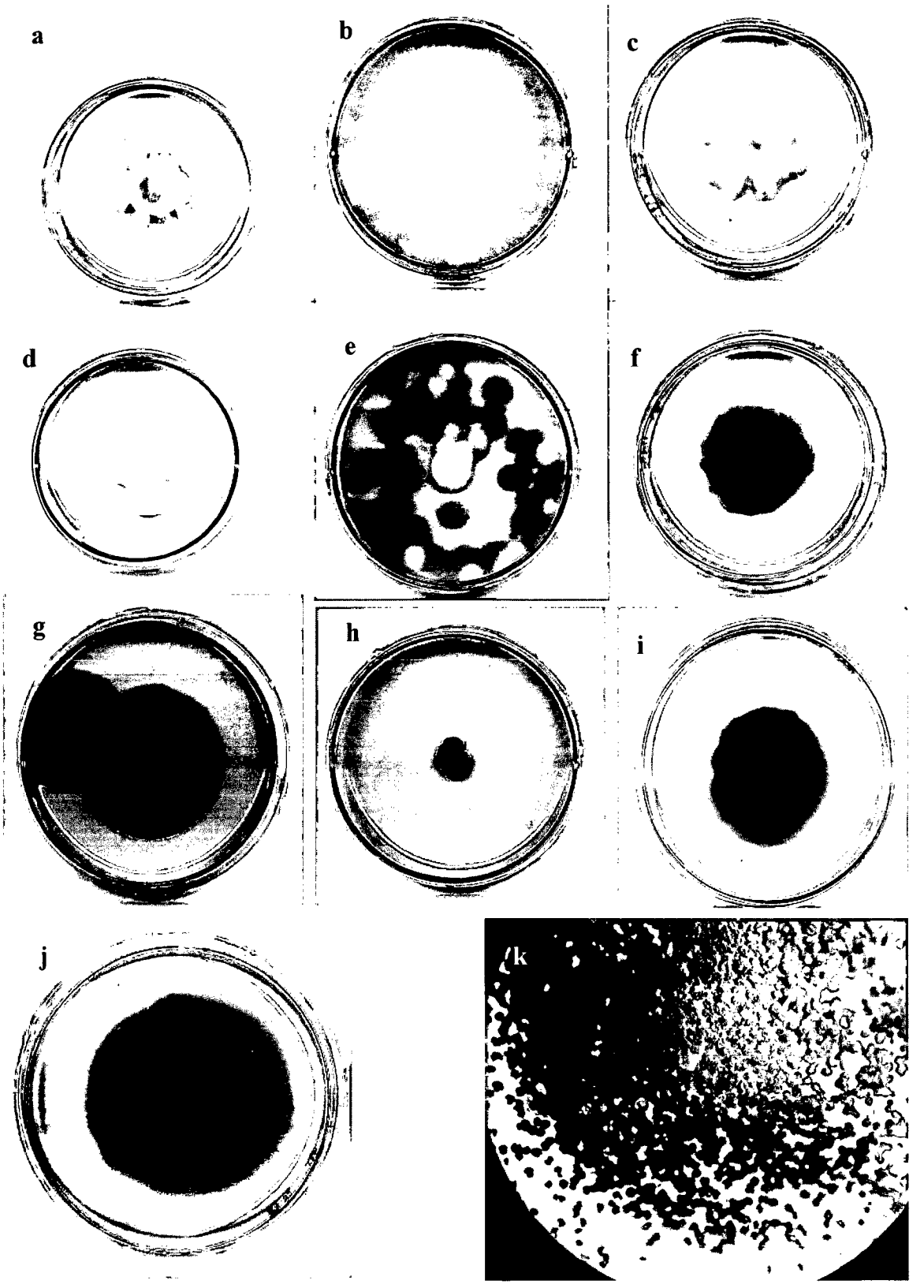


Fig. 14 Pure colonies of fungi sourced from mosquito and other insects (7-day-old culture on PDA) a. *Fusarium oxysporum;* b. *Mucor* sp.; c. *Fusarium solani ;* d. *Aspergillus* sp. 2; e. *Fusarium* sp.; f. *Aspergillus* sp. 3; g. *Aspergillus* sp. 1; h. *Penicillium* sp.; i. *Aspergillus niger* isolate D4; j. *A. niger* isolate D2; k. *A. niger* D4 under stereomicroscope, 4x.

hyphae. *Conidiophores* mononematous, aseptate, unbranched, verruculose, thin-walled, hyaline, 600-800 x 4-5 µm with pyriform, 10-14 µm in diam. vesicles. *Conidiogenous cells* uniseriate, phialidic, discrete, bottle-shaped, covering upper 3/4 of the vesicle, 3-4 x 1-3 µm. *Conidia* catenate, globose to subglobose, smooth to verruculose, thick-walled, 1- celled, yellowish green, 1-2 µm in diameter.

Specimen examined: On a Long Horned Beetle, from Taleigao Plateau, Goa, India, 28°C, 9.09.2004; leg. DP, direct isolation, Culture No. GU/BOT/NIMR/, Slide No. D3.

4. *Aspergillus* sp. 2 (Fig. 14d)

Entomogenous hyphomycete. *Colonies* moderately growing on PDA with a diam. of 3.4 cm in 7 days. Light yellow coloured colony with yellow granular nature centrally periphery showed folds somewhat radially striate and marginally zonate, consisting of a thin but almost continuous layer of sclerotia which shade quickly from white through golden yellow to cinnamon rufous. Reverse of the colony yellow coloured, folds seen radiating from centre, margin smooth. *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 2-4 µm wide hyphae. *Conidiophores* mononematous, coarsely roughened, heavy walled, aseptate, unbranched, hyaline, 450-500 x 5-7.5 µm, with globose vesicles having a diam. of 30-60 µm. *Conidiogenous cells* phialidic, on biseriate metulae, covering entire vesicle, 10-15 x 5-6 µm. *Metulae* cylindrical, smooth, brown, 7-9 x 2.2-3.5 µm. *Conidia* catenate, elliptical very rough, thick-walled, 1-celled, black, 3.5- 4.4 µm diameter.

Specimen examined: On an infested *Anopheles* adult, from NIMR, Panaji, Goa, India, 24°C, 19.11.2004; leg. DP, direct isolation, Culture No.GU/BOT/NIMR/, Slide No. D8.

5. *Aspergillus* sp. 3 (Fig. 13f; Fig. 14f)

Moderately growing colony with growth of 4.1 cm diameter in 7 d; granular dark green fruiting body, shiny exudate droplets in the centre, periphery white with fringed root-like margin, reverse side of the colony light yellow. Conidial heads radiate, pale greenish yellow, later becoming light to dull brown. *Conidiophores* hyaline, up to 1410-2990 x 80.4-101 µm mostly rough-walled. Vesicles subglobose, 20-40 µm in diam. *Phialides* often directly borne on the vesicle or on metulae, usually measuring 10-15 x 3-5 µm. *Metulae* 8-12 x 4-5 µm. Conidia ellipsoidal when young, globose to subglobose when mature, 4.5-8 µm in diam, green, smooth to finely rough-walled.

Specimen examined: On a Long Horned Beetle, from Farmagudi, Goa, India, 28°C, 19.11.2004; leg. DP, direct isolation, Culture No. GU/BOT/NIMR/, Slide No. D9.

6. *Fusarium oxysporum* Schltdl., *Fl. berol.* (Berlin) **2**: 139 (1824) **(Fig. 13e; Fig. 14a)**

Moderate growing colony ; 4.2 cm colony diameter, irregular colony margin, pinkish white aerial mycelia, colony margin pink, wooly, colony reddish purple on reverse side, agar with a reddish halo of 1 cm around the colony. Conidiophores are short, single, lateral monophialides in the aerial mycelium. Conidiogenous cell 6.3-8.5 x 3-4 µm. Macroconidia fusiform, slightly curved, pointed at the tip, 1-3 septate, basal cells pedicellate, 23-54 × 3.0-4.5 µm. Microconidia borne on simple phialides arising laterally on the hyphae abundant, never in chains, aseptate, ellipsoidal to cylindrical, straight or often curved, 5-12 × 2.3-3.5 µm. Chlamydospores terminal or intercalary, hyaline, smooth or rough-walled, 5-13 µm diameter.

Specimen examined: From larval bait of *Ae. aegypti* used in backwaters of Carambolim, Goa, India, 30°C, 03.04.2005; leg. DP, isolation using baits, Culture No. GU/BOT/NIMR/, Slide No. D1.

7. *Fusarium solani* (Mart.) Sacc., *Michelia* **2**(no. 7): 241-301 (1881) **(Fig. 13b-d; Fig. 14c)**

Entomogenous hyphomycete. *Colonies* on PDA circular, moderately growing colony with growth of 4.5 cm diam. in 7 d white cottony/wooly, margin irregular, rhizoid-like, reverse of the colony with vinaceous pigmentation, agar light orangish. *Mycelium* composed of smooth, septate, freely branched, thin-walled, hyaline, 2-4 µm wide hyphae. *Conidiophores* arising laterally from aerial hyphae. Monophialides long and slender mostly with a rather distinct collarette. *Conidiogenous cells* phialidic, discrete, narrow cylindrical, hyaline 25-40 x 2-4 µm. *Macroconidia* produced on shorter conidiophores which soon form sporodochia, usually with short, blunt apical and indistinctly pedicellate basal cells, solitary, wedge shaped, moderately curved, ends blunt, smooth, 1-4-septate, hyaline, 12-32 x 3-4.5 µm; *Microconidia* globose, aseptate, produced on elongate, sometimes verticillate conidiophores, 4-5 x 3-4 µm. Chlamydospores hyaline, globose or ovoid frequent, smooth or rough-walled, singly or in pairs, terminal or in lateral branches, intercalary or in chains 3-5 µm diameter.

Specimen examined: From bait of *Anopheles* larvae set in backwaters of Saat Mancher, Carambolim, Goa, India, 30°C, 03.04.2005; leg. DP, isolation using baits, Culture No. GU/BOT/NIMR/, Slide No. D7.

8. *Fusarium* sp. (Fig. 14e)

Slow growing colony with a diam. of 1.2-1.5 cm in 7 days. Colony surface white to pinkish brown in colour, wooly, reverse side light brown with pale yellowish periphery. Aerial mycelia sparse. Conidiophores loosely branched, with short, often swollen phialides, 10-18 × 4-5 µm. Microconidia broadly lunate to reniform either almost uniformly rounded at both ends on submerged hyphae, mostly 1-celled, 4.5-9 x 2-3 µm, rarely 1-septate. Macroconidia formed abundantly in masses on poorly or

well developed sporodochia, typically widest in the upper third, central part minutely curved or nearly straight, with a slightly beaked and pointed distal and a slightly pedicellate proximal end; typically with one median septum, 12.5-15.5 x 3-3.5 μm. Chlamydospores mostly intercalary, spherical, 6-12 μm diam., smooth-walled, single or in chains.

Specimen examined: From bait of *Anopheles* larvae set in rice fields of Banastarim, Goa, India, 30°C, 03.04.2005; leg. DP, isolation using baits, Culture No. GU/BOT/NIMR/, Slide No. D5.

Fusarium is an anamorphic genus affiliated with Hypocreales (Ascomycetes). The sexual stages (teleomorphs) of the species are in *Gibberella* and *Nectria*. *Fusarium* is characterized by production of slimy, hyaline, septate, canoe-shaped conidia known as macroconidia. Some species also produce distinctly different conidia in the aerial mycelium referred to as microconidia. According to the species and/or the ecological situation, either macroconidia or microconidia may dominate on natural substrate. Chlamydospores are also produced by some species. Modern taxonomy of *Fusarium* is based mostly on cultural characters (Booth, 1971; Gerlach & Nirenberg, 1982; Nelson *et al.*, 1983).

9. *Penicillium* sp. (Fig. 13a; Fig. 14h)

Entomogenous hyphomycete. *Colonies* show slow growth, bluish grey with 1.5 cm diam. in 7 days. Cartilagenous, folded granular colony with exudate droplets. Reverse side yellow highly folded margins crinkled with peripheral lappet like folds directed towards the centre. *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 1.8-2.2 μm wide hyphae, penicilli terverticillate. *Conidiophores* mononematous, smooth, septate, branched terminally, thin-walled, hyaline, 31.5-72.5 x 2-2.4 μm; Metulae solitary or in verticils of 2-5, smooth, 9.2 -19.6 × 1-1.7 μm.

Phialides solitary or in verticils of 3-11, ampulliform, smooth, 6.7-9.2 × 5.8-7.6 µm; collula narrow some with a length up to 2.5 µm; *Conidia* dry, catenate, globose or ellipsoidal, aseptate, smooth, thin walled, 1-1.7 µm in diameter.

Specimen examined: From bait of *Anopheles* set in backwaters of Saat Mancher, Carambolim, Goa, India, 30°C, 02.04.2005; leg. DP, isolation using baits, Culture No. GU/BOT/NIMR/, Slide No. D6.

4.1.3 Sourcing of mosquito-pathogenic fungi from culture collection and taxonomy:

The identity of the seven fungal isolates with known mosquito larvicidal activity obtained for the present study from GUFCC was reconfirmed using standard taxonomic keys and monographs. They were further identified up to species level wherever possible, the others were identified at the level of genera. The fungi are described in detail below.

1. *Gliocladium roseum* Bain. 1907. *Bull. Soc. Mycol. Fr.* **23**: 111 (1907) **(Fig. 15.b and Fig. 15.c)**

Entomogenous hyphomycete. *Colonies* on CMA circular, pale creamy white, with smooth hairy margin, growth median, attaining a diam. of 3-4 cm in 7 days, slimy, concentric rings of wet globules of conidia after initial 1-2 cm floccose growth with thick mycelial strands bearing short conidiophores and wet heads of conidia; reverse of the colony colourless (Fig.16.a). *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 2-3 µm wide hyphae. *Conidiophores* two types: primary conidiophores of *Verticillium*-type, and secondary conidiophores of penicillate type, mononematous, smooth, sometimes pitted, septate, branched, thin-walled, hyaline, mostly 100-150 µm in length, 60-200 µm long, 2-3 µm at the base, 3-4 µm at the middle and 2-3 µm at the base of penicillia, the base of the conidiophore narrows.

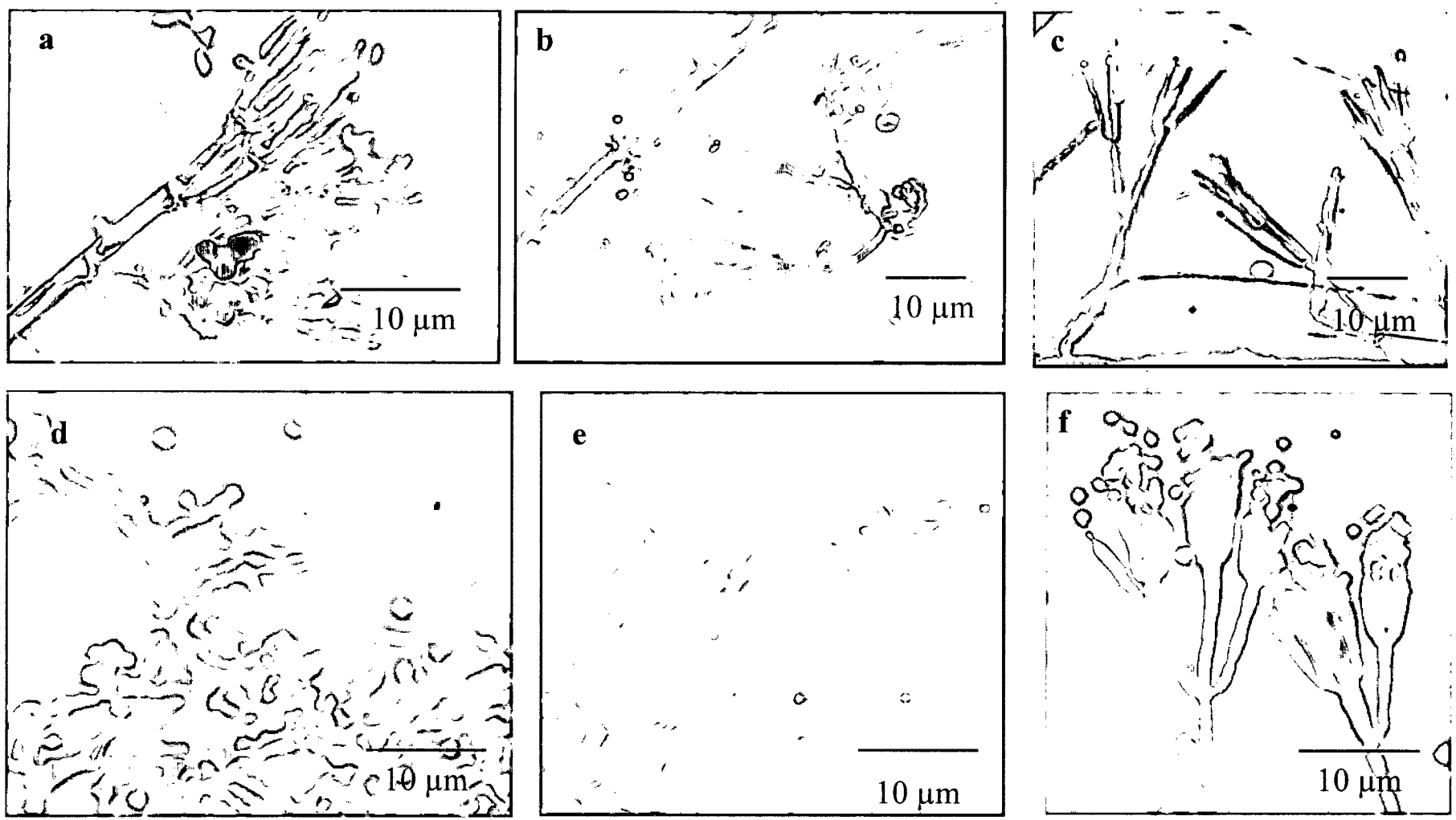


Fig. 15 Microscopic characters of mosquito-pathogenic fungi sourced from GUFCC a. *Gliocladium* sp. (100X); b. *Gliocladium roseum* isolate GUFCC 5039 (40X Phase contrast); c. *Gliocladium roseum* isolate GUFCC 5040 (40X); d. *Trichoderma atroviride* (100X); e. *Trichoderma* sp. (100X) ;f. *Penicillium citrinum* (100X).

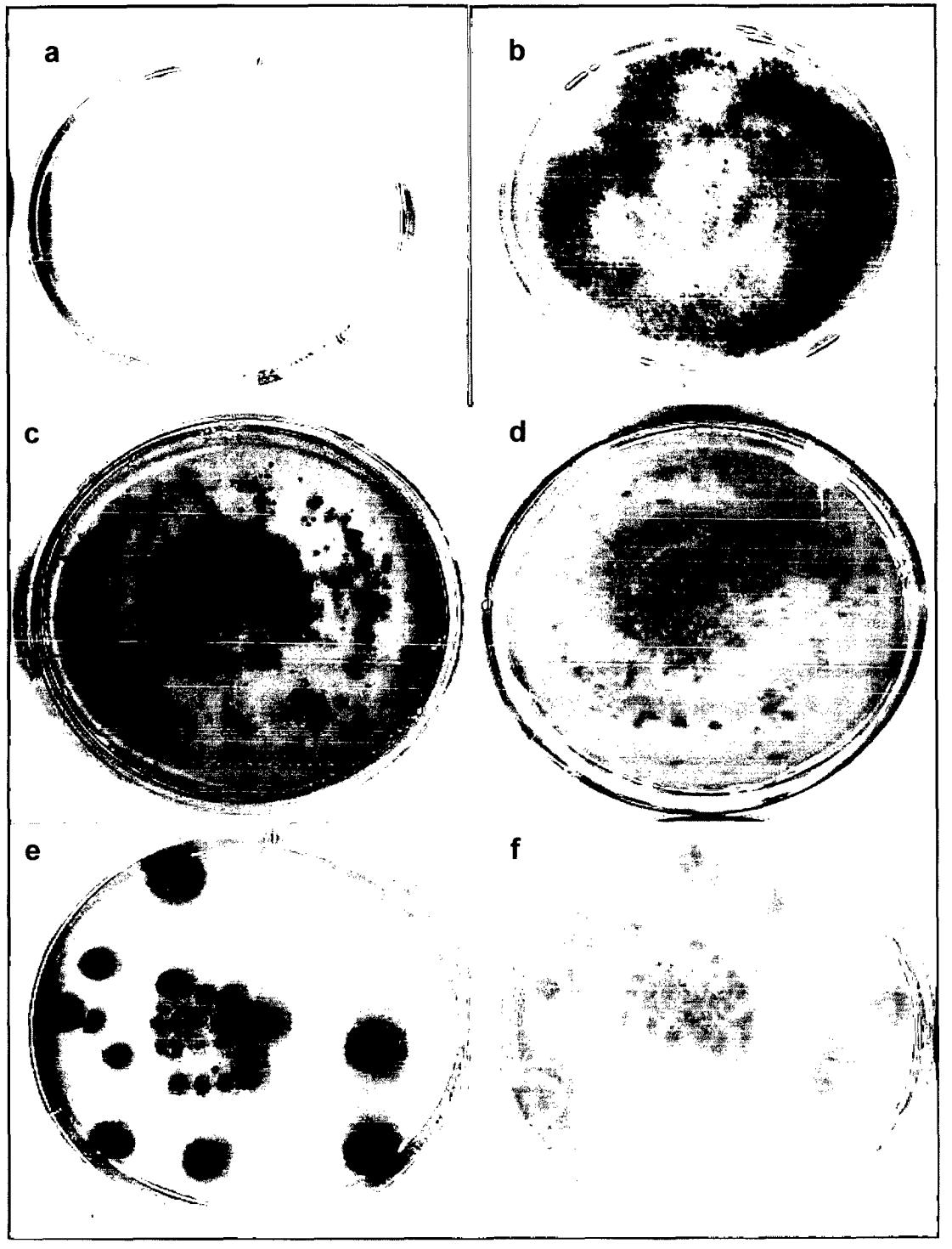


Fig. 16 Pure colonies of fungi sourced from GUFCC (7-day-old cultures) a. *Gliocladium roseum* isolate GUFCC 5040 on MEA b. *Trichoderma atroviride* on CMA c. *Trichoderma* sp. isolate on MEA d. Reverse side of *Trichoderma* sp. isolate on MEA e. *Penicillium citrinum* on MEA f. Reverse of *P. citrinum* on MEA.

Conidiogenous cells phialidic, discrete, 10-25 x 2-3 µm tapering towards the tip. *Conidia* slimy, solitary, asymmetrically navicular, hyaline, 4-7 x 3-4 µm.

Specimen examined or source: i) On aphids on fresh leaves of *Chromolaena odorata,* scrub jungle, Pernem, Goa, India, 29°C, 29.06.1999, leg. KP, direct isolation, Culture No. GUFCC 5039 ii) On aphids on fresh leaves of *Cassia tora,* scrub jungle, Pernem, Goa, India, 28°C, 29.06.1999, KP, direct isolation, Culture No. GUFCC 5040.

Typified by *Gliocladium penicilloides* Corda, the genus *Gliocladium* Corda can be seen as a counterpart of *Penicillium* with slimy conidia. Characteristic features of the species are densely penicillate conidiophores with slimy heads of 1-celled hyaline or brightly pigmented conidia. Along with penicillate conidiophores, there are also predominantly verticillate conidiophores. However, typically asymmetrical conidia of such members help in distinguishing them from *Verticillium* spp. Following Domsch *et al.* (1980) the isolates were identified as *Gliocladium roseum* Bain. 1907.

2. *Gliocladium* sp. (Fig. 15.a)

Entomogenous hyphomycete. *Colonies* on CMA circular, creamy white, flat, smooth hairy margin, growth median attaining a diam. of 3-4 cm in 7 days, slimy, with concentric rings of wet globules of conidia, reverse of the colony colourless; *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 2-3 µm wide hyphae. *Conidiophores* mononematous, smooth, sometimes pitted, septate, branched, thin-walled, hyaline, mostly 100-150 (60-200) µm long, 2-3µm wide at the base, 3-4 µm at the middle, 2-3 µm at the base of penicillia, with base of the conidiophore abruptly narrow. *Conidiogenous cells* phialidic, discrete, 16-25 x 2-3 µm tapering towards the tip. *Conidia* slimy, solitary oval with twisted proximal tip, 4-6 x 2-3 µm.

Specimen examined or source: On aphids on fresh leaves of *Chromolaena odorata,* scrub jungle mixed with paddy fields and plantation, Curchorem, Goa, India, 28°C,

22.07.1999, leg. KP, direct isolation, Culture No. GUFCC 5044.

3. *Penicillium citrinum* Thom 1910. *U.S.D.A. Bur. Anim. Ind.Bull.***118**: 61. **(Fig. 15.f)**

Entomogenous hyphomycete. Colony characters were studied on MEA, colonies were consistently restricted and were generally smaller than on Czapek, plain, with heavy sporulation, with reverse typically pale yellow. *Colonies* were circular, green with white periphery, wavy or smooth, hairy margin, median growth, attaining a diam. of 2.8-3.3 cm in 7 days, powdery, with watery droplets, with concentric rings, reverse of the colony pale yellow (Fig. 16.e,f). *Mycelium* composed of smooth, septate, branched, hyaline, 2 µm wide hyphae. *Conidiophores* mononematous, finely verrucose, pitted, septate, rarely branched terminally, 100-300 x 2-2.5 µm penicilli biverticillate; *metulae* asymmetrical, verruculose, hyaline 15-40 x 3-4 µm, swollen at the distal tip 5-8 µm. *Conidiogenous cells*, phialidic, discrete, bottle- shaped, with a distinct neck, hyaline, 8-10 x 2-2.5 µm. *Conidia* dry, catenate, globose, verrucose, 1-celled and green, 3 µm.

Specimen examined or source: On an *Anopheles* sp. 2nd larva, from curing water at construction site, Panaji, Goa, India, 30°C, 20.04.1999, leg. KP, direct isolation, Culture No. GUFCC 5072.

4. *Penicillium* sp.

Entomogenous hyphomycete. *Colonies* on CMA circular, green, median growth, attaining a diam. of 2.5-3 cm in 7 days, with scanty superficial hyphae, with watery droplets, reverse of the colony colourless. *Mycelium* composed of smooth, septate, branched, thin-walled, hyaline, 1.6-2.4 µm wide hyphae. *Conidiophores* mononematous, smooth to verruculose, septate, branched terminally, thin-walled, hyaline, 100-300 x 1.6-2.4 µm; *metulae* asymmetrical, hyaline, verruculose, 7-18 x 3-4 µm, swollen into a vesicle 5-6 µm diam. *Conidiogenous cells* phialidic, discrete,

attached laterally onto vesicle, closely packed, 5-11 x 2.4-3.2 µm. *Conidia* dry, catenate, globose to subglobose, aseptate, verruculose, 2-3µm in diam.

Specimen examined or source: On 12 immature stages of homopteran insects on *Ixora coccinea*, mixed farm, Kasaragod, Kerala, India, 29°C, 24.05.1999, leg. KP, moist chamber incubation, Culture No. GUFCC 5077.

5. *Trichoderma atroviride* P. Karst., *Bidr. Känn. Finl. Nat. Folk* **51**: 363 (1892) **(Fig. 15.d)**

Entomogenous hyphomycete. *Colonies* on CMA circular, hyaline submerged hyphae with, superficial patches of green conidial mass and rhizoidal margin, growth fast attaining a diam more than 9 cm in 7 days, slimy, fruit-bodies in patches, initially white later changing to green, with watery exudates, reverse of the colony colourless (Fig. 16.b). *Mycelium* composed of smooth, septate, freely branched, thin-walled, hyaline, 4-7 µm wide hyphae. *Conidiophores* mononematous, coarse with tubercles, septate, profusely branched, thin-walled, hyaline, 30-40 x 2-3 µm. *Conidiogenous cells* phialidic, discrete, rough, 5-7x 3-3.5 µm with a very narrow beak like distal tip. *Conidia* globose, smooth, forming sticky balls of 10-15 on each conidiogenous cell, green, 2-3 µm diam.

Specimen examined or source: On an *Anopheles* sp. 3rd instar larva, curing water at construction sites, Cuncolim, Goa, India, 26°C, 24.09.1999, leg. KP, direct isolation, culture No.GU/BOT/ICMR/C37, Slide No. C37. GUFCC 5103.

6. *Trichoderma* sp. (Fig. 15.e; Fig.16.c,d)

Entomogenous hyphomycete. *Colonies* on MEA circular, hyaline submerged hyphae with green patches of conidial mass and rhizoidal margin, fast growing, attaining a diam. of 9 cm in 7 days, slimy, fruit-bodies in bush like patches, initially white later changing to green, with watery exudates, reverse of the colony colourless (Fig.16.c,d).

Mycelium composed of smooth, septate, freely branched, thin-walled, hyaline, 4-7 µm wide hyphae. *conidiophores* mononematous, coarse with tubercles, septate, profusely branched, thin-walled, hyaline, 40-70 x 2-3 µm. *Conidiogenous cells* phialidic, discrete, rough, hyaline, 5-7x 3-3.5 µm with a very narrow beak like distal tip. *Conidia* globose, smooth, sticky forming balls of 10-15 conidia over each conidiogenous cell, green, 3 µm.

Specimen examined or source: On an *Anopheles* sp. 3rd instar larvae, curing water at construction sites, Porvorim, Goa, India, 30°C, 23.04.1999, leg. KP, direct isolation, culture No. GUFCC 5088.

4.2 Analysis of larvicidal potential and bio-efficacy of fungal isolates:

During the present study 51 bioassays were carried out for the screening, bio-efficacy of the pathogenic fungi and their metabolites and 3 bioassays for studying safety to non-target organisms.

4.2.1 Preliminary testing of fungi for larvicidal potential:

Preliminary testing of the five fungal isolates screened for larvicidal activity using doses in the range of 2 x 10^6 spores/ml - 20 x 10^6 spores/ml against mosquito larvae revealed percent mortalities in thc range of 16-100% (Table 8).

Bioassays with *Gliocladium* species

1. Bioassays with *Gliocladium* sp. isolate GUFCC 5044 resulted in 100% mortality in *Cx. quinquefasciatus* 3rd instar larvae on 24 h exposure to conidial dose of 2.7 x 10^6 spores/ml prepared from 14-day old culture.

2. *G. roseum* isolate GUFCC 5040 from 14-day old culture caused 92.8% mortality in *An. stephensi* 3rd instar larvae on 72 h exposure. *Cx. quinquefasciatus* 3rd instar larvae

were comparatively less susceptible to this isolate with mortality of 71.9% when exposed to the same conidial dose of 10.52 x 10^6 spores/ml for 72 hours. However, with the increase in time of exposure from 24 to 72 hours the mortality in *Culex* larvae increased by 25.2% i.e. from 46.7% to 71.9%. When compared it was 4 fold higher than the 6.1% increase observed in *An. stephensi* larvae (Fig. 17).

In the exposed *An. stephensi* larvae the mortality difference was not significant between the 5 replicates when exposure time was increased from 24 h to 72 h ($F = 1.091$, $p = 0.394$) (Table 9), whereas the difference in mortality was significant in the case of *Cx. quinquefasciatus* 3rd instar larvae ($F = 9.333$, $p = 0.014$) (Table 10).

Bioassays with the *G. roseum* isolate grown for 21 days using a higher conidial dose of 18 x 10^6 spores/ml against *Cx. quinquefasciatus* 3rd instar larvae resulted in 62.2% mortality on 72 h exposure whereas in 4th instar larvae 60% mortality was observed at the same dose and exposure time. Hence the 3rd instar larvae were little more susceptible than the 4th instar *Cx. quinquefasciatus* larvae (Fig.18). It was noted that the surviving 3rd instar larvae did not enter the 4th instar and the exposed 4th instar did not pupate except 1/60 which later emerged as an adult. They appeared stout and sluggish suggesting that their further growth was arrested. The larvae in the control were however active, healthier, feeding voraciously and developed into pupae from which healthy adults emerged.

Bioassays with *Trichoderma* species

1. In the bioassay with *T. atroviride* cultured for 14 d, 70% mortality in *Cx. quinquefasciatus* and 25% in *An. stephensi* 3rd instar larvae was observed on 72 h exposure to conidial dose of 6.9 x 10^6 spores/ml and 11.6 x 10^6 spores/ml respectively.

Table 8: Preliminary screening of fungi for their pathogenicity to mosquito larvae.

Sr. No.	Fungal sp.	Dose ($nx10^6$ spores /ml)	Mosquito sp.	Instar	Percent mortality Time (h)		
					24	48	72
1.	*Gliocladium* sp. isolate GUFCC 5044 (14d)	2.7	*Culex quinquefasciatus*	3rd	100		
2.	*Gliocladium roseum* isolate GUFCC 5040 (14d)	10.52	*Cx. quinquefasciatus*	3rd	46.7	56.7	71.9
3.	*G. roseum* isolate GUFCC 5040 (14d)	10.52	*Anopheles stephensi*	3rd	86.7	89.5	92.8
4.	*G. roseum* isolate GUFCC 5040 (21d)	18	*Cx. quinquefasciatus*	3rd	36.7	47.4	62.2*
5.	*G. roseum* isolate GUFCC 5040 (21d)	18	*Cx. quinquefasciatus*	4th	30	43.2	60*
6.	*Penicillium citrinum* isolate GUFCC 5072 (14d)	10	*Cx. quinquefasciatus*	3rd	78	100	
7.	*Trichoderma atroviride* isolate GUFCC 5103 (14d)	6.9	*Cx. quinquefasciatus*	3rd	28	46	70

8.	*T. atroviride* isolate GUFCC 5103 (14d)	11.6	*An. stephensi*	3rd	8.3	20	25
9.	*Trichoderma* sp. isolate GUFCC 5088 (14d)	14.19	*Cx. quinquefasciatus*	3rd	5.3	9.3	16

*Corrected mortality. In the rest mortality in the control was nil.

Table 8 A Larvicidal bioassay of *Gliocladium roseum* GUFCC 5040 (21d spore suspension) vs *Culex quinquefasciatus* III & IV instar larvae. (n=20)

Cx. quinquefasciatus III instar larvae					*Cx. quinquefasciatus* IV instar larvae			
Control		Experimental			Control		Experimental	
1	2	1	2	3	1	2	1	2
00	00	00	00	00	00	00	00	00
00	00	08	07	07	00	00	04	08
01	01	11	09	10	01	02	09	10
01	02	15	11	13	03	04	14	13

Table 8 B

Observed and corrected percent mortality in *Culex quinquefasciatus* III & IV instar larvae exposed to *Gliocladium roseum* GUFCC 5040 (21d spore suspension). (n=20)

Time (h)	*Cx. quinquefasciatus* III instar larvae			*Cx. quinquefasciatus* IV instar larvae		
	Control	Experimental		Control	Experimental	
		Actual mortality	*Corrected mortality		Actual mortality	*Corrected mortality
0	0	0		0	0	
24	0	36.67		0	30	
48	5	50	47.4	7.5	47.5	43.2
72	7.5	65	62.2	17.5	67.5	60.6

Table 9: ANOVA table to ascertain the significance of larvicidal activity of conidial suspension (14 d) of *Gliocladium roseum* isolate GUFCC 5040 against *Anopheles stephensi* 3rd instar larvae with respect to different time intervals

Anova Table

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Treatments	2	66.667	33.333	1.091	0.394
Error	6	183.333	30.556	-	-
Total	8	-	-	-	-

Coefficient of Variation = 6.142

Differences in mortality at different Treatments found to be Non Significant

Table 10: ANOVA table to ascertain the significance of larvicidal activity of conidial suspension (14 d) *Gliocladium roseum* isolate GUFCC 5040 against *Culex quinquefasciatus* 3rd instar larvae with respect to different time intervals

Anova Table

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Treatments	2	1088.889	544.444	9.333	0.014*
Error	6	350.000	58.333	-	-
Total	8	-	-	-	-

Coefficient of Variation = 12.970

Treatments found Significant at 5% level of Significance

F cal = F calculated value; F prob = F probability; * = significant

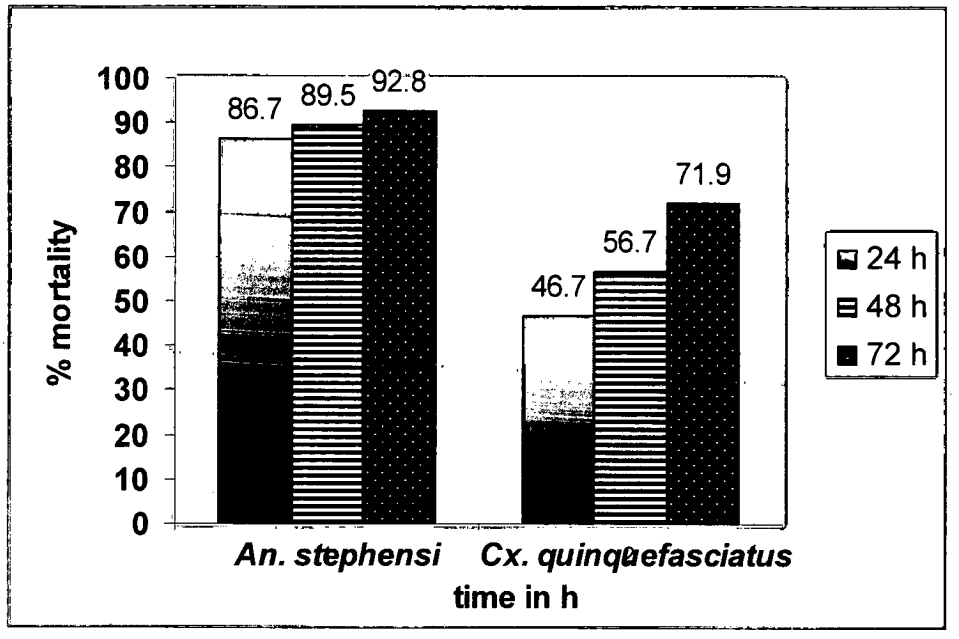


Fig. 17 Susceptibility of 3rd instar mosquito larvae to *Gliocladium roseum* (isolate No. GUFCC 5040).

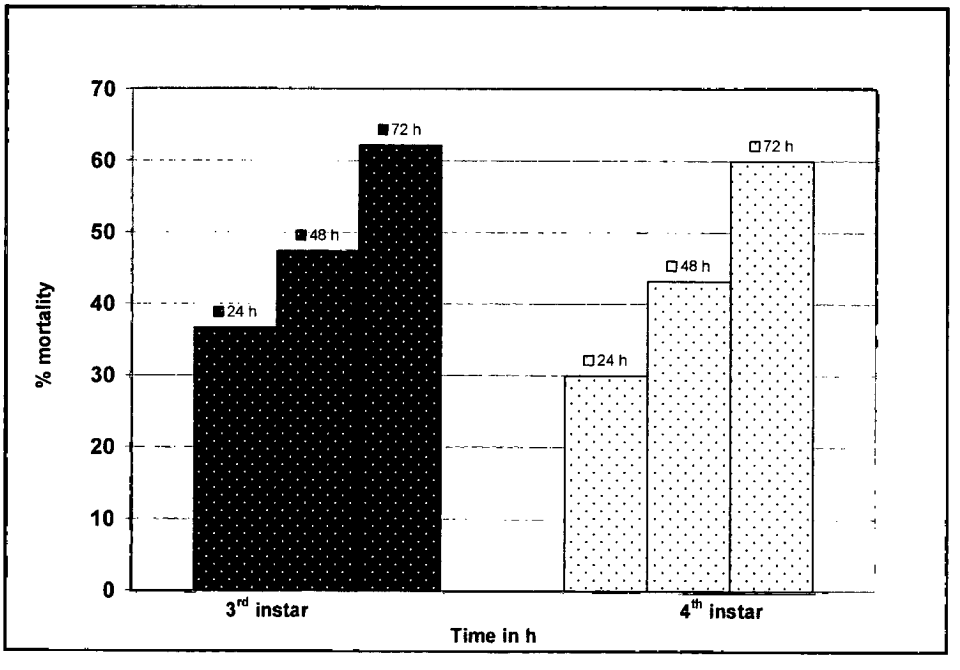


Fig. 18 Mortality caused by *G. roseum* (isolate GUFCC 5040) in *Cx. quinquefasciatus* 3rd and 4th instar larvae.

The surviving larvae entered the 4th instar but appeared weak. The larvae in the control were however active, robust, feeding voraciously, and developed normally.

2. Comparatively 14 d conidial suspension of *Trichoderma* sp. isolate GUFCC 5088 caused a low mortality of 16% in the *Cx. quinquefasciatus* larvae at a conidial dose of 14.19 x 10^6 spores/ml on 72 h exposure.

Bioassays with *Penicillium* species

1. The susceptibility of 3rd instar larvae of *Cx. quinquefasciatus* was 100% on 48 h exposure to 14 d conidial suspension of *P. citrinum* at a dose of 10 x 10^6 spores/ml.

4.3 Mode of invasion of active fungi in mosquito larvae:

4.3.1 Light microscopic study:

Mode of invasion of three fungal isolates, *Gliocladium* sp. isolate GUFCC 5044, *T. atroviride* and *P. citrinum* studied in the 3rd instar larvae of test vector species by light microscopy, histological techniques and cryostat sectioning revealed different routes of invasion.

The *Cx. quinquefasciatus* larvae exposed to an active dose of 2.7 x 10^6 spores /ml of *Gliocladium* sp. isolate GUFCC 5044 were observed for 8 h on hourly basis. After six hours the mycelia emerged from the cuticle and by 16 h the whole body was engulfed in the mycelia (Fig. 19 a,b). The larvae on 24 h exposure were showing profuse mycelial growth from the dorsal side, the anal segment and the respiratory siphon. The degree of emergence of mycelia differed in different larvae (Fig. 19 c). Though there was profuse mycelial growth in some larvae, death did not occur immediately. The control larvae were normal without any growth (Fig. 19 d).

Cx. quinquefasciatus larvae were exposed for 48 h to an active dose of 6.9 x 10^6 spores/ml of *T. atroviride*. Under stereomicroscope the alimentary canal of *Cx.*

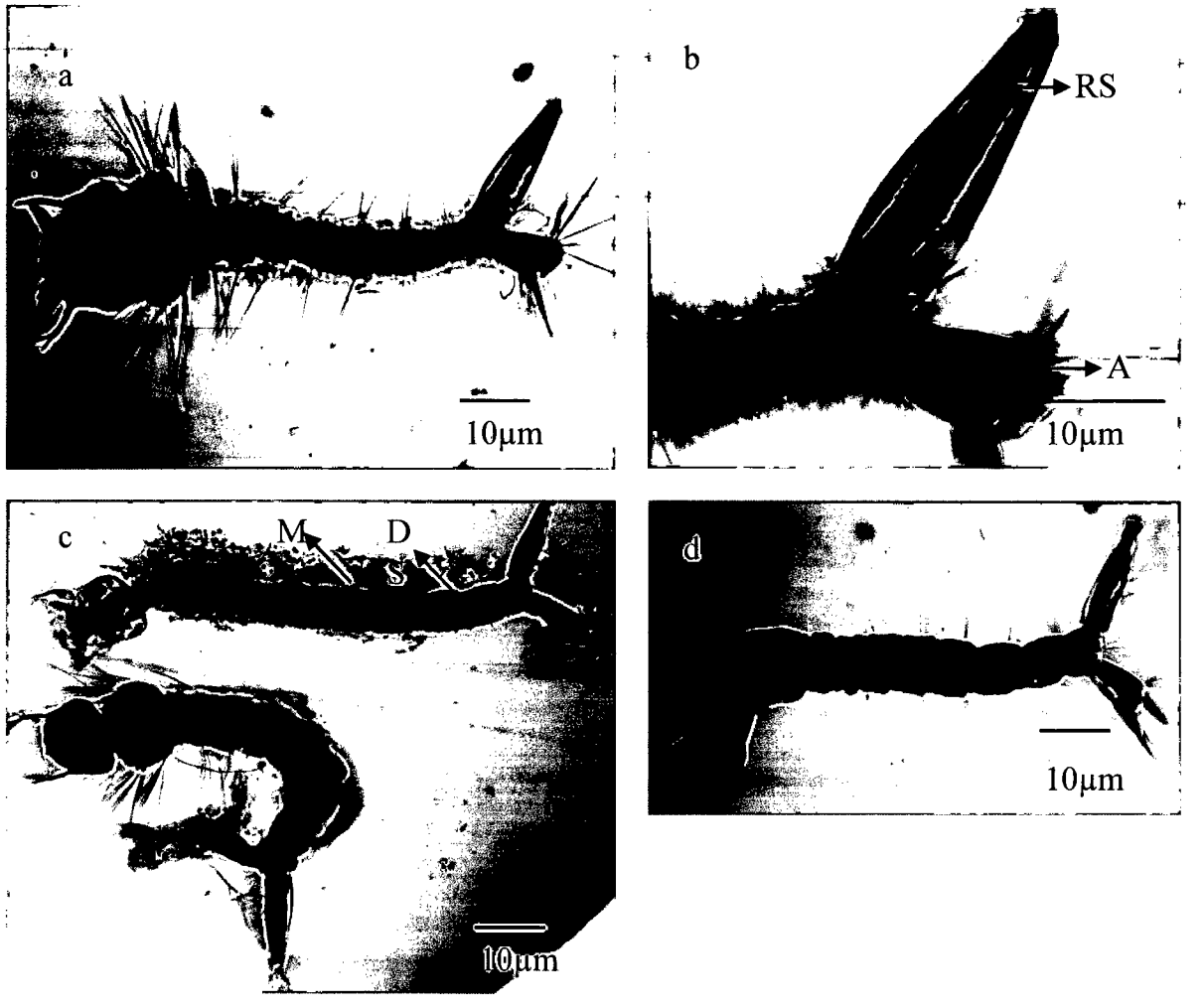


Fig. 19 Mode of invasion of *Gliocladium* sp. isolate GUFCC 5044 in *Culex quinquefasciatus* 3rd instar larvae a. 16 h exposed larvae(10x). b. Magnified view of posterior abdominal segments of 16 h exposed larvae (45x). c. 24 h exposed larvae(10x). d. Control (10x). A= Anus, DS= dorsal surface, M= mycelium, RS= respiratory siphon.(scale bar = 10 µm).

quinquefasciatus larvae appeared packed with green coloured matter probably green spores of *T. atroviride*. Dead larvae had distorted body with head separated from the thorax. Similar observations were seen in case of *An. stephensi* larvae exposed to an active dose of 11.6 x 10^6 spores/ml.

In *Cx. quinquefasciatus* larvae exposed to *P. citrinum* at an active dose of 10 x 10^6 spores/ml the transparent body also appeared packed with green conidia (Fig. 20 b) when observed at 24 hours. At 48 h hyphae emerging out from the cuticle could be observed (Fig. 20.C). Also a number of ciliates could be observed in the haemocoel indicating gut rupture leading to their escape from the gut into the haemocoel.

In *An. stephensi* larvae exposed for 24 h to *P. citrinum* at an active dose of 20 x 10^6 spores/ml intense blackening of the body was observed indicating melanin production. The head hung on one side of the body in dead larvae through an extended thorax in exposed *Anopheles* and *Culex* larvae.

In case of *Ae. aegypti* exposed for 72 h to *P. citrinum* at an active dose of 80 x 10^6 spores/ml in the dead larvae, no blackening or distortion of thorax was observed.

4.3.2 Histological study:

4.3.2A Histopathological light microscopic study of mode of action of *Gliocladium* sp. isolate GUFCC 5044

Mode of action of *Gliocladium* sp. isolate GUFCC 5044 in *Cx. quinquefasciatus* larvae was studied by taking histological sections of the larvae exposed for 24 h to a dose of 2.7 x 10^6 spores /ml. The haemocoel of the larva was ramified with fungal mycelia and the organ tissues were disrupted at 24 h exposure (Fig. 21.b). The cuticle also showed the presence of hyphae.

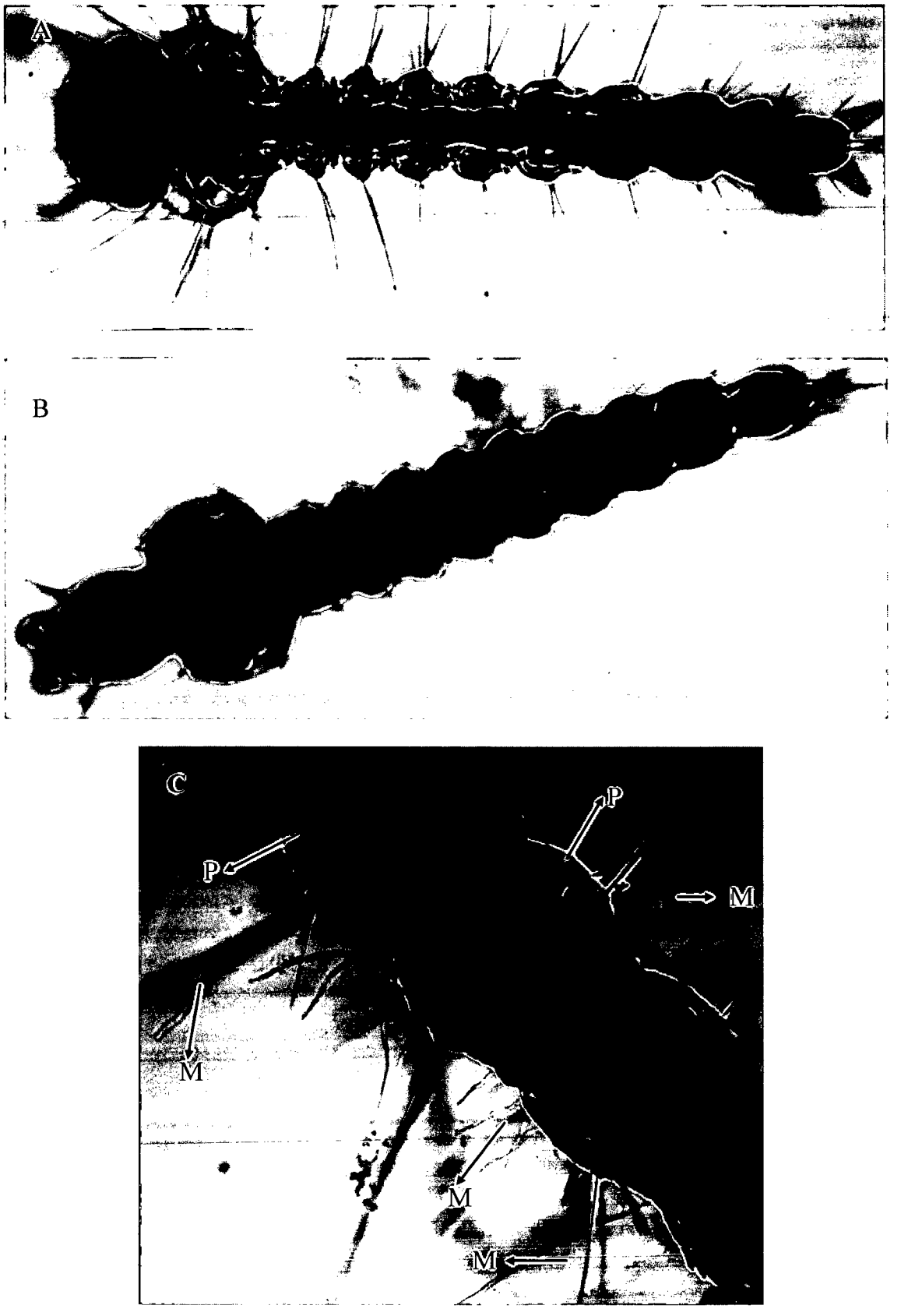


Fig. 20 Mode of action of *Penicillium citrinum* on *Culex quinquefasciatus* 3rd instar larva A. Control larva (16x). B. Experimental larva-Gut filled with *P. citrinum* conidia (16x). C. Magnified view of experimental larva (64x). M= Mycelium emerging from the cuticle, P=Protozoan ciliates.

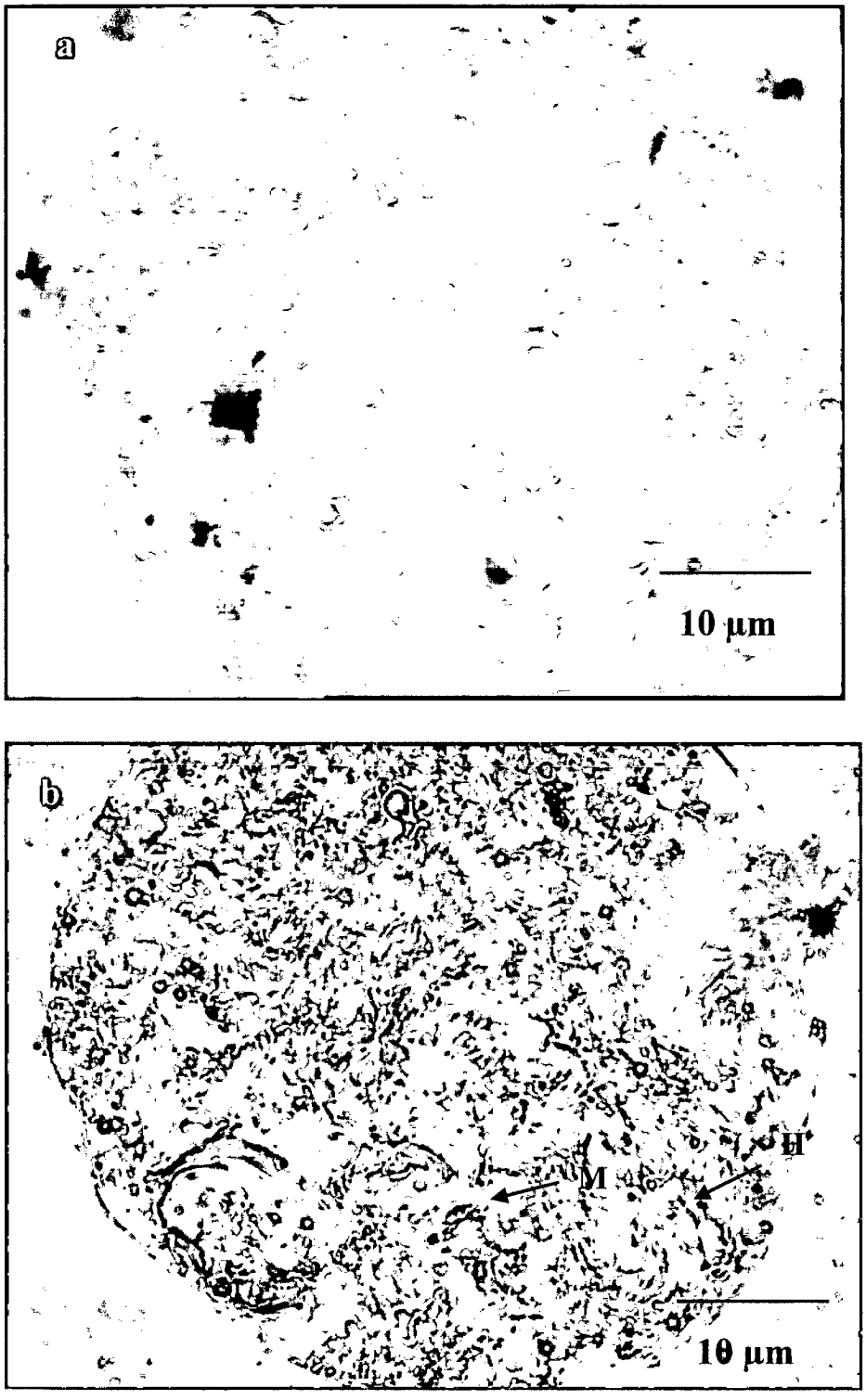


Fig. 21 T. S of *Cx. quinquefasciatus* larva a. Control b. Larva exposed to *Gliocladium* sp. (isolate GUFCC 5044) H=haemocoel, M=Mycelium. 100x (scale bar = 10 µm).

4.3.2B Light Microscopic study on mode of action of *T. atroviride* by cryostat sectioning:

The dissected gut of *Cx. quinquefasciatus* larvae which were exposed to *T. atroviride* showed the conidia in the chyme. Light microscopy revealed the presence of the conidia in the foregut and midgut region of the alimentary canal (Fig. 22.b) in the longitudinal sections (L.S) of *Cx. quinquefasciatus* larvae. It was also noted that the tissue had taken up blue colour while the conidia remained unstained (Fig. 22 b, c) from the foregut up to the midgut, probably they were coated with some secretions which lacked affinity to the stain, many had assumed crescent shape, slight flattening of the conidia observed maybe due to mechanical pressure, a tendency of conidia to remain more closer to the gut epithelium observed. Though conidia remained for 48 h in the gut, germination or hyphal formation did not happen.

4.3.3 Scanning Electron Microscopy studies:

Scanning Electron Microscopy was carried out to study the mode of action of *P. citrinum* in 3rd instar larvae of *Cx. quinquefasciatus* exposed for 48 h and *Ae. aegypti* exposed for 72 hours. SEM of *Cx. quinquefasciatus* control (Fig. 23a, b) and experimental larvae showed that the latter had shriveled up (Fig. 23 d) and also the head was hanging from the thoracic region (Fig. 23e). The presence of *P. citrinum* conidia on the respiratory siphon and the anal gills in the exposed *Cx. quinquefasciatus* was also observed (Fig. 24a). Comparatively a few conidia were observed adhering to the body in case of *Ae. aegypti*. SEM of the digestive system of *Cx. quinquefasciatus* larva exposed to show the gut contents revealed that the gut was filled with conidia (Fig. 25A). Also the formation of appressoria on the conidia was observed (Fig. 25B). SEM of faecal pellets of *Cx. quinquefasciatus* and *Ae. aegypti*

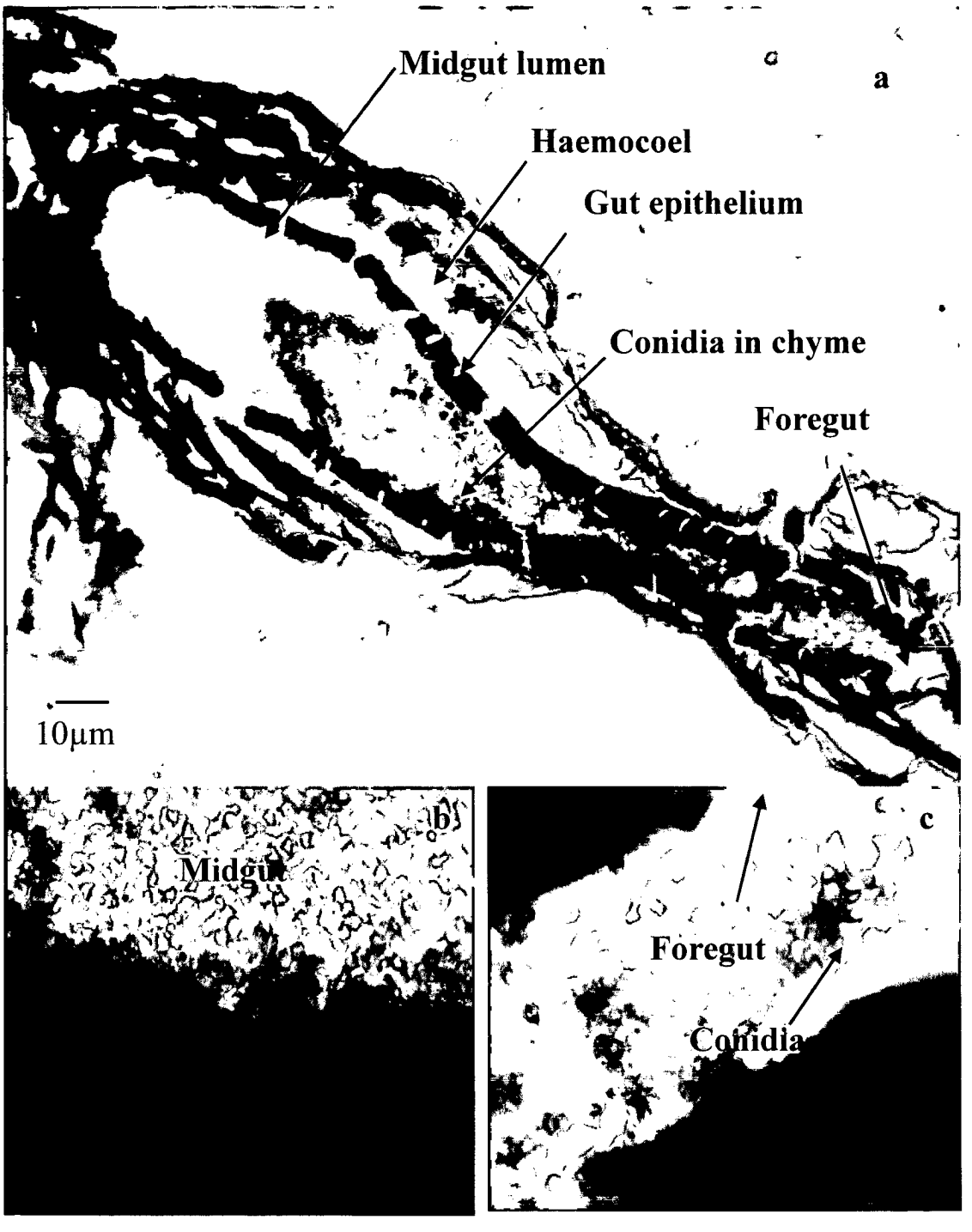


Fig. 22 Photomicrographs of L.S of *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure to *T. atroviride* conidia. a. L.S view of the entire gut, 10x (bar = 10 µm) b. View of the midgut with conidia more towards the gut epithelium, 40x (scale bar = 10 µm). c. Magnified view of the foregut alongwith the conidia, 40x (scale bar = 10 µm).

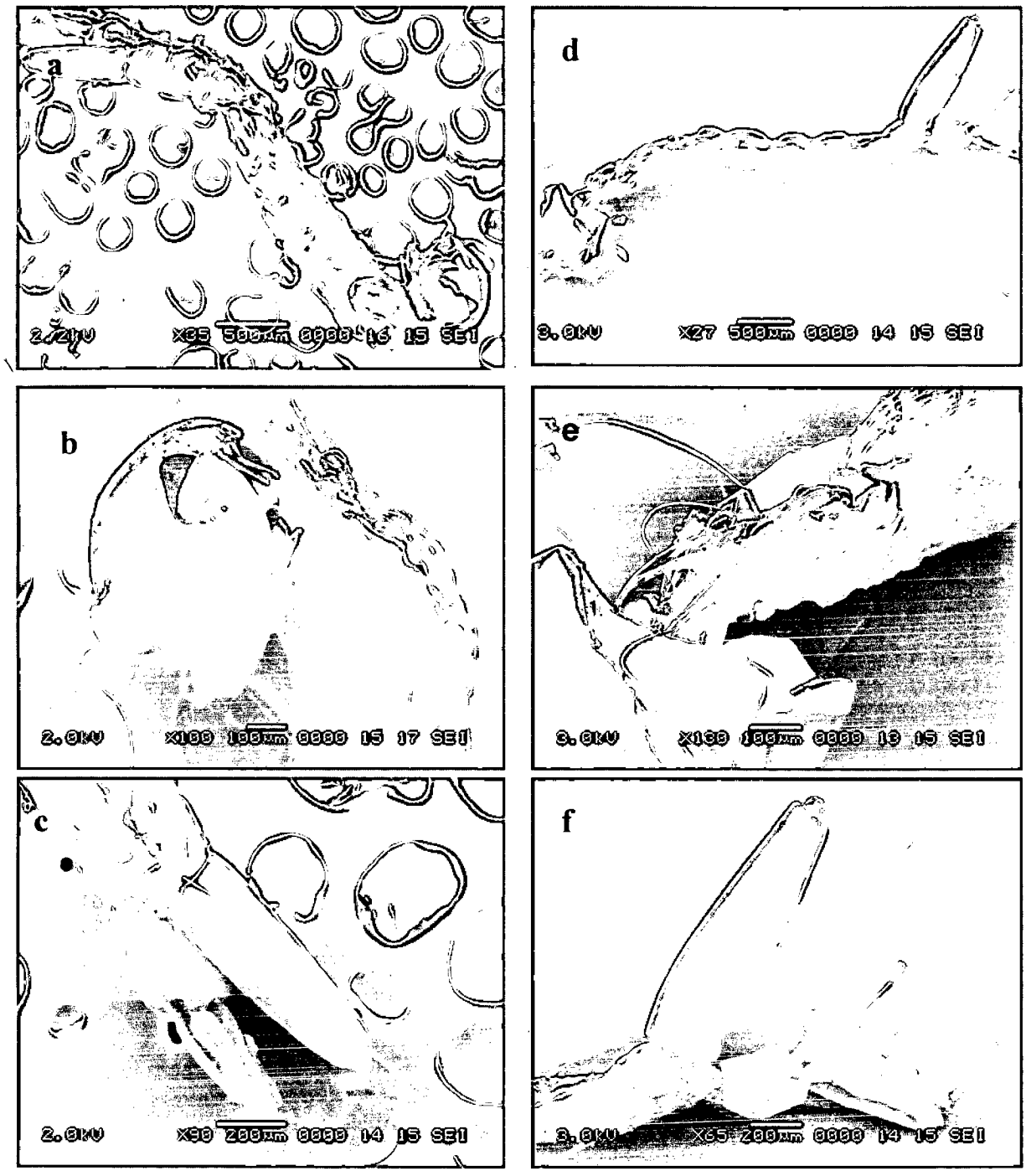


Fig. 23 a-f SEM of *Cx. quinquefasciatus* 3rd instar larva a-c Control larva a. Whole mount (Scale bar = 500 µm) b. head (Scale bar = 100 µm) c. vii and viii abdominal segments with respiratory siphon and gills (Scale bar = 200 µm) d-f *Culex* Larva exposed to *P. citrinum* for 48 h d. Whole mount of exposed larva (Scale bar = 500 µm) e. Head hanging from thoracic region (Scale bar = 130 µm) f. Last three abdominal segments with respiratory siphon and gills (Scale bar = 200 µm).

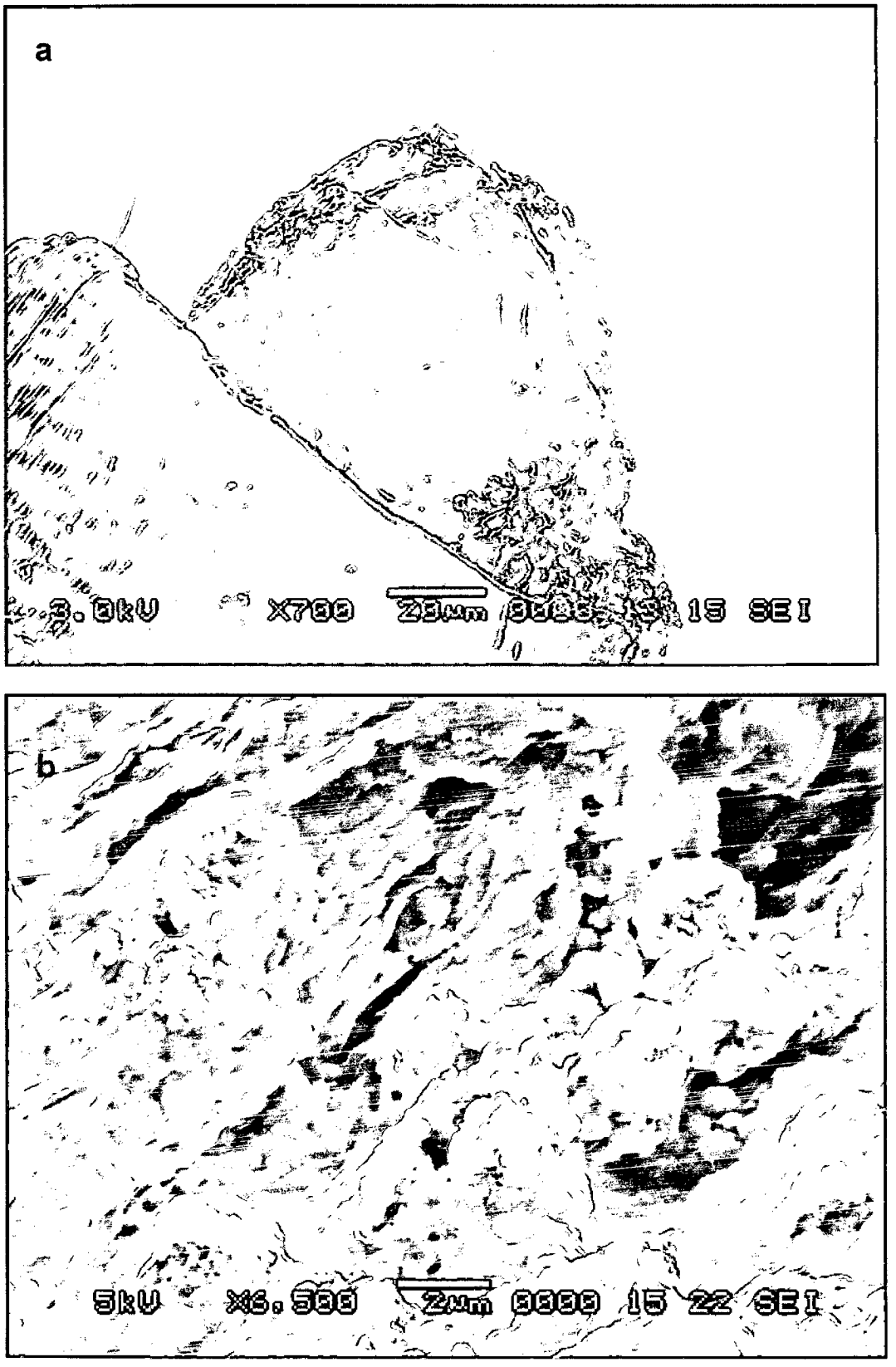


Fig. 24 a. SEM of respiratory siphon of *Cx. quinquefasciatus* 3rd instar larva exposed to *P. citrinum* for 48 h also seen are conidia of *P. citrinum* (Scale bar = 20 µm) b. SEM of faecal pellet of *Ae. aegypti* showing conidia of *P. citrinum* enveloped in peritrophic membrane. (Scale bar = 2 µm).

Fig. 25 SEM of *Cx. quinquefasciatus* 3rd instar larva exposed to *P. citrinum* for 48 h. A. Digestive system of *Cx. quinquefasciatus* larva exposed to show the conidia of *P. citrinum* within the gut (Scale bar = 10 µm). B. Magnified view of exposed digestive tract with conidia of *P. citrinum* showing formation of appressoria. a = appressoria, c = conidia, d = digestive system (Scale bar = 2µm).

larvae showed the presence of intact conidia of *P. citrinum* enveloped in peritrophic membrane (Fig. 24b).

4.3.4 Fate of fungal spores:

The viability experiments showed that after being held for more than two hours in the gut of *Cx. quinquefasciatus* larvae, the conidia of *T. atroviride* remained viable. They were viable prior to ingestion, the viability was confirmed by running larvicidal bioassay. The conidia retained their viability ensuing ingestion and excretion. After holding in distilled water for 24 h when plated on CMA, the conidia germinated.

In the *Cx. quinquefasciatus* 3rd instar larva, the mode of action of different species of fungi differed. In *Gliocladium* sp. isolate GUFCC 5044 cuticle of *Cx. quinquefasciatus* seemed to be the invasion route while in *T. atroviride* and *P. citrinum* the invasion seemed to be through the gut of the larva.

4.4 Subculturing of three isolates and loss of activity in case of *Gliocladium* sp. (isolate GUFCC 5044) and *T. atroviride*:

In our study involving three promising isolates viz., *Gliocladium* sp. isolate GUFCC 5044, *T. atroviride* and *P. citrinum* chosen for mode of action and bio-efficacy, loss of activity was noticed in the first two after subculturing for six months while the last candidate remained stable retaining its virulence after subculturing for two years. Hence this candidate was selected to study bio-efficacy, bio-safety to NTO, study of metabolites and enzymes.

4.5 Analysis of bio-efficacy of promising isolate:

The bio-efficacy of the most promising isolate *P. citrinum* isolate GUFCC 5072 was assessed by performing bioassays against 3rd instar larvae of the three vector species

in 250 ml water. The spore dose range of *P. citrinum* giving 0-100% mortality was 2 x 10^6 - 10 x 10^6 spores/ml in case of *Cx. quinquefasciatus* larvae, 4.58 x 10^6 - 20.02 x 10^6 spores/ml in case of *An. stephensi* larvae and 3.44 x 10^6- 89.44 x 10^6 spores/ml in *Ae. aegypti* larvae. The relation between various doses and corresponding mortalities is graphically represented in Fig. 26.

No significant mortalities (0-1.6%) were seen in the controls. The LD_{50} and LD_{90} values obtained against *An. stephensi* were 17.13 x 10^6 and 70.46 x 10^6 spores/ml at 24 h and 2.55 x10^6 and 61.969 x 10^6 spores/ml respectively at 48 h.

The difference in mortalities was not significant between the 3 replicates/dose ($F = 0.533$, $p = 0.60$), while it was highly significant in between 5 doses ($F = 28.20$, $p < 0.01$). The effect of time on mortality was also significant ($F = 5.403$, $p < 0.05$) (Table 11).

The LD_{50} and LD_{90} values obtained against *Cx. quinquefasciatus* larvae were 0.002 x 10^6 and 125767.468 x 10^6 spores/ml at 24 h and 0.041 x 10^6 and 22.732 x 10^6 spores/ml respectively at 48 h.

The difference in mortalities in *Cx. quinquefasciatus* larvae was not significant between the 3 replicates/dose ($F = 3.127$, $p = 0.075$), it was also not significant between the 4 doses ($F = 1.328$, $p = 0.305$). The effect of time on mortality was also not significant ($F = 0.256$, $p = 0.621$) (Table 12).

The LD_{50} and LD_{90} values obtained against *Ae. aegypti* larvae were 80.13 x 10^6 and 1818.701 x 10^6 spores/ml at 24 h and 66.03 x 10^6 and 6221.951 x 10^6spores/ml at 48 h respectively.

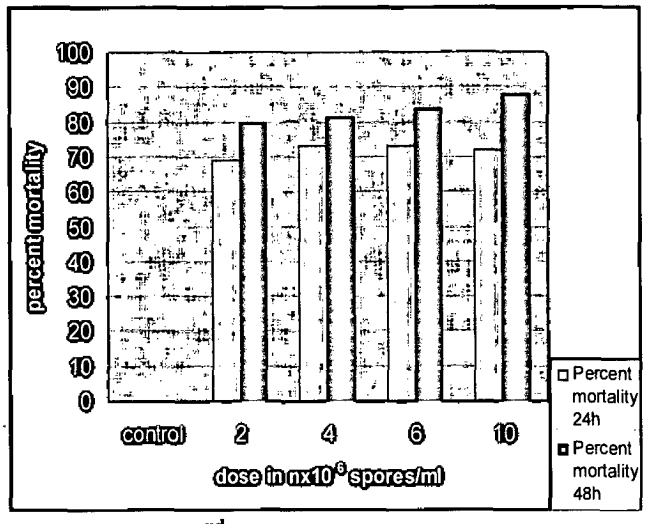
The difference in mortalities in *Ae. aegypti* larvae was not significant between the 3 replicates/dose ($F = 1.571$ $p = 0.230$), while it was highly significant between the 4 doses ($F = 55.755$, $p = 0.00$). The effect of time on mortality was highly significant ($F = 37.860$, $p = 0.00$) (Table13).

In *An. stephensi* the F cal is greater than critical difference (CD) values at 5% and 1% for the different doses used (F cal = 28.201; >CD 5% = 7.592 and > CD 1%=10.399). Whereas with regards to the time intervals (F cal = 5.403; > CD 5% = 4.801 and < CD 1%=6.577). This confirms that in the test mortality difference is highly significant with regard to doses whereas it is significant with regard to time.

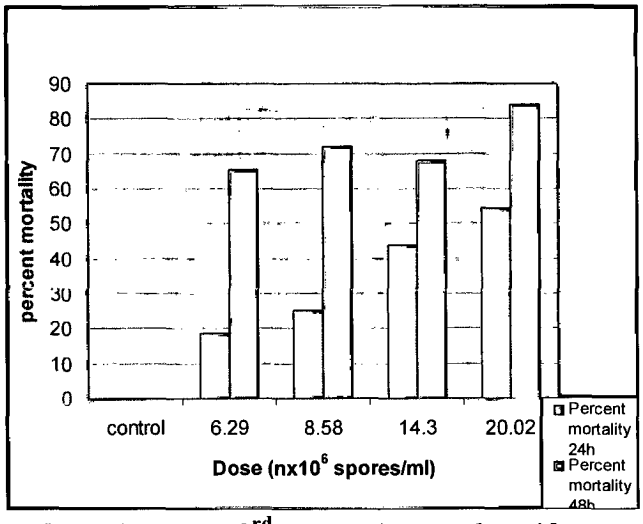
In *Cx. quinquefasciatus* the F cal is lesser than critical difference (CD) values at 5% and 1% for the different doses used (F cal = 1.328 <CD 5% = 13.837; and < CD 1% = 19.204), the second variable i.e. time also shows a similar trend (F cal =0.256 <CD 5% = 9.784and < CD 1% = 13.579) indicating the test is not significant with regard to doses and time.

In *Ae. aegypti*, the F cal exceeds critical difference (CD) values at 5% and 1% for the different doses used (F cal = 55.755; >CD 5% = 5.906; and > CD 1% = 8.027). It also exceeds with regards to the time intervals (F cal = 37.860; >CD 5% = 5.114 and > CD 1% = 6.951). This confirms that the test is highly significant with regard to doses as well as time.

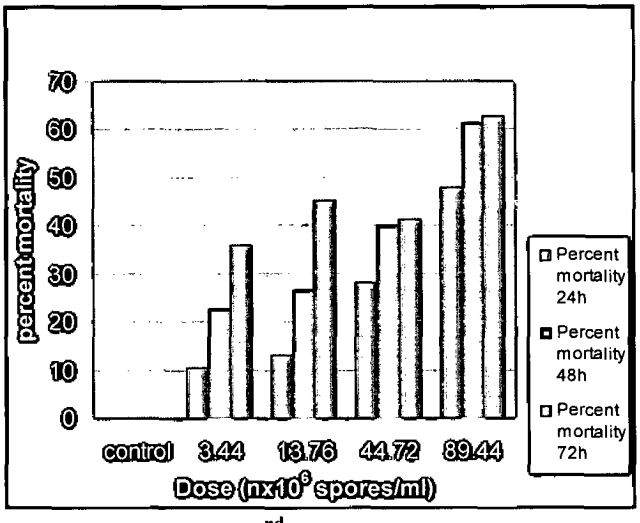
In *Cx. quinquefasciatus* larvae, *P. citrinum* caused 69.3% average percent mortality on 24 h exposure and 80% average percent mortality on 48 h exposure at a dose of 2 x 10^6 spores/ml. In *An. stephensi* larvae, it caused 30.6% average percent mortality on 24 h exposure and 66.6% average percent mortality on 48 h exposure at a dose of 4.58 x 10^6 spores/ml whereas in *Ae. aegypti* larvae it caused 10.6% average percent mortality on 24 h exposure and 22.7 % average percent mortality on 48 h exposure and 36% average percent mortality on 72 h exposure at a dose of 3.44 x 10^6 spores/ml. The highest dose that each vector species was exposed to were 10 x 10^6 spores/ml in case of *Cx. quinquefasciatus* larvae, in *An. stephensi* larvae 20.02 x 10^6 spores/ml and *Ae. aegypti* larvae a dose of 89.44 x 10^6 spores/ml. This dose in *Cx. quinquefasciatus*



a) *P. citrinum* vs. 3rd instar *Cx. quinquefasciatus* larvae.



b) *P. citrinum* vs. 3rd instar *An. stephensi* larvae.



c) *P. citrinum* vs. 3rd instar *Ae. aegypti* larvae.

Fig. 26: Dose response relationship of *P. citrinum* vs. 3rd instar mosquito larvae.

Table 11 A. Main Bioassay of *P. citrinum* v/s *Anopheles stephensi* III larvae

Dose conc.(spores/ml)	Mortality/replicate at different time intervals (n=25)							
	24h				48h			
	R1	R2	R3	$\bar{x}$	R1	R2	R3	$\bar{x}$
6.29×10^6	05	05	04	05	19	15	15	16
8.58×10^6	09	05	05	06	16	18	20	18
14.3×10^6	10	11	12	11	16	16	19	17
20.02×10^6	12	15	14	14	23	19	21	21
0 Control	00	00	00	00	00	00	00	00

Table 11 B. Main Bioassay of *P. citrinum* v/s *Culex quinquefasciatus* III larvae

Dose conc.(spores/ml)	Mortality/replicate at different time intervals (n=25)							
	24h				48h			
	R1	R2	R3	$\bar{x}$	R1	R2	R3	$\bar{x}$
2×10^6	18	20	14	17	18	22	20	20
4×10^6	19	24	12	18	24	24	13	20
6×10^6	12	22	21	18	16	23	24	21
10×10^6	15	18	21	18	19	24	23	22
0 Control	00	00	00	00	00	00	00	00

Table 11 C. Main Bioassay of *P. citrinum* v/s *Aedes aegypti* III larvae

Dose conc.(spores/ml)	Mortality/replicate at different time intervals (n=25)											
	24h				48h				72h			
	R1	R2	R3	$\bar{x}$	R1	R2	R3	$\bar{x}$	R1	R2	R3	$\bar{x}$
3.44×10^6	2	4	2	3	6	5	6	6	7	12	8	9
13.76×10^6	2	6	2	3	5	8	7	7	11	13	10	11
44.72×10^6	8	7	6	7	10	9	11	10	10	9	12	10
89.44×10^6	12	10	14	12	14	16	16	15	15	16	16	16
0 Control	00	00	00	00	01	00	00	00	01	00	01	00

Table 11D: Analysis of variance values for bioassay of *P. citrinum* vs. *An. stephensi* 3rd instar larvae exposed for 24 and 48h.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
replications	2	41.732	20.866	0.533	0.596
Treatments	9	5019.263	557.696	14.238	0.000**
Factor A	4	4418.421	1104.605	28.201	0.000**
Factor B	1	211.621	211.621	5.403	0.032*
A X B	4	389.221	97.305	2.484	0.080
Error	18	705.046	39.169	-	-
Total	29	-	-	-	-

Coefficient of Variation = 13.356

Critical Difference Values		
-	CD 5%	CD 1%
Factor A	7.592	10.399
Factor B	4.801	6.577
Treatments/(A x B)	10.736	14.707

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

Table 12: Analysis of variance values for bioassay of *P. citrinum* vs. *Cx. quinquefasciatus* 3rd instar larvae exposed for 24 and 48h.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Replications	2	780.798	390.399	3.127	0.075
Treatments	7	562.366	80.338	0.644	0.714
Factor A	3	497.316	165.772	1.328	0.305
Factor B	1	31.910	31.910	0.256	0.621
A X B	3	33.141	11.047	0.088	0.965
Error	14	1747.762	124.840	-	-
Total	23	-	-	-	-

Coefficient of Variation = 17.638

Critical Difference Values

-	CD 5%	CD 1%
Factor A	13.837	19.204
Factor B	9.784	13.579
Treatments/(A x B)	19.569	27.159

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

Table 13: Analysis of variance values for bioassay of *P. citrinum* vs. *Ae. aegypti* 3rd instar larvae exposed for 24, 48and 72h

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Replications	2	114.667	57.333	1.571	0.230
Treatments	11	9334.667	848.606	23.259	0.000**
Factor A	3	6102.667	2034.222	55.755	0.000**
Factor B	2	2762.667	1381.333	37.860	0.000**
A X B	6	469.333	78.222	2.144	0.089
Error	22	802.667	36.485	-	-
Total	35	-	-	-	-

Coefficient of Variation = 16.625

Critical Difference Values

-	CD 5%	CD 1%
Factor A	5.906	8.027
Factor B	5.114	6.951
Treatments/(A x B)	10.229	13.903

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

Table 13 B: Probit equations to depict larvicidal activity of *P. citrinum* vs. Mosquito III instar larvae

Mosquito sp.	Time (h)	Equation Y=a +bx	LD_{50}= n x 10^6 spores/ml (95% Confidence Limits Lower-Upper)	LD_{90} n x 10^6 spores/ml (95% Confidence Limits Lower-Upper)
Aedes aegypti	24	Y=-1.801 +0.946x	80.247 (37.399-743.208)	1818.701 (321.164-2832278.205)
	48	Y=-1.181 +0.649x	66.03 (26.508-2474.447)	6221.951 (512.495-1.483E11)
	72	Y=-0.457 +0.495 x	8.384	3267.859
Anopheles stephensi	24	Y=-2.564+2.078x	17.130 (12.779-40.341)	70.462(33.625-2147.000)
	48	Y=-0.376+0.925 x	2.551 (0.000-5.030)	61.969 (26.921-5.961E10)
Culex quiquefasciatus	24	Y=0.444 +0.164 x	0.002	125767.468
	48	Y=-0.648 +0.467x	0.041	22.732

larvae caused average mortalities of 72% and 88% on 24 h and 48 h exposure respectively; in *An. stephensi* larvae 54.66 % and 84 % and in *Ae. aegypti* larvae 48 %, 61.33 % and 62.7 % average percent mortality on 24 h, 48 h and 72 h exposure respectively showing thereby that *Cx. quinquefasciatus* larvae were most susceptible to *P. citrinum* followed by *An. stephensi* and *Ae. aegypti* in decreasing order of susceptiblility.

4.6 Assessment of metabolites of effective fungi:

4.6A. Screening of secondary metabolites of *T. atroviride* in cell free extract for larvicidal activity:

Bioassays with metabolites from the cell free extract of *T. atroviride* cultured for 10 d against *Cx. quinquefasciatus* larvae with doses of 1 µl/ml, 10 µl/ml and 100 µl/ml caused percent mortalities of 14%, 22% and 76% respectively after 24 h exposure and 24%, 46% and 99%, respectively after 48 h exposure. Control mortality ranged from 0-6.66%. LC_{50} for 24 h was 26.36 µl/ml and LC_{50} for 48 h was 5.886 µl/ml (Table 14). Mortality difference was significant within the 5 replicates/dose ($F = 6.227$, $p = 0.002$), while it was highly significant between the 3 doses ($F = 244.342$, $p < 0.001$). The significance in mortality was high with increase in exposure time from 24 h to 48 h ($F = 20.061$, $p = 0.000$) (Table15). The F cal value exceeds critical difference (CD) values at 5% and 1% for the different doses used (F cal = 244.342; >CD 5% = 3.876; and > CD 1% = 5.287). It also exceeds with regards to the time intervals (F cal = 20.061; >CD 5%=3.165 and > CD 1% = 4.317). This confirms that the test is highly significant with regards to doses as well as time.

4.6B. Screening of extra cellular metabolites of *Penicillium* spp. for larvicidal activity:

The extra cellular metabolites in 14 d and 20 d cell free culture filtrates of *P. citrinum* isolate and *Penicillium* sp. isolate GUFCC 5077 tested against 3rd instar larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* larvae at a dose of 200 µl/ml did not show any larvicidal activity on 24 h and 48 h exposure.

4.6C. Screening of secondary metabolites of *Penicillium* spp. extracted in organic solvent for larvicidal activity:

The crude extract with methanol of *Penicillium* sp. isolate GUFCC 5077 14 d culture containing intracellular metabolites when screened for larvicidal activity against *Cx. quinquefasciatus*, *An. stephensi* and *Ae. aegypti* 3rd instar larvae did not cause any mortality at a dose of 5 µl/ml. A higher dose of 10 µl/ml failed to cause mortality in the latter.

On screening *P. citrinum* metabolites from 14 d culture extracted in methanol, 73% mortality in *An. stephensi* and 17.5% mortality in *Ae. aegypti* 3rd instar larvae was obtained on 24 h exposure to a dose of 4 µl/ml and 5 µl/ml respectively (Fig. 27.A). Further in *An. stephensi* larvae on 48 h exposure a mortality of 83% was obtained and in *Ae. aegypti* larvae 37.5% mortality was observed.

The difference in mortalities in *An. stephensi* larvae *P. citrinum* metabolites from 14 d culture was not significant between the 3 replicates/dose ($F=0.371$, $p=0.699$), it was also not significant between the 3 doses ($F=2.872$, $p=0.103$). The effect of time on mortality was however significant ($F=5.306$, $p<0.05$) (Table 16).

P. citrinum metabolites from 20 d culture resulted in 60% mortality in *An. stephensi* and 5% mortality in *Ae. aegypti*, on 24 h exposure to a dose of 4µl/ml and 5µl/ml

Table 14: Probit equations and susceptibility of *Cx. quinquefasciatus* larvae to metabolites of *T. atroviride* isolate GUFCC 5103 (X is the log concentration of metabolites of *T. atroviride* in µl/ml).

Time (h)	Probit equation	LC50 (µL/ml)	95% Confidence Interval		χ2
			Lower Bound	Upper bound	
24	-1600+1.126X	26.36	0.408	1.844	1.205
48	-1.057+1.373X	5.88	0.551	2.195	1.375

Table 15: The significance of larvicidal activity of metabolites of *T. atroviride* against *Cx. quinquefasciatus* larvae obtained by two factor ANOVA.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Replications	4	430.048	107.512	6.227	0.002*
Treatments	5	16531.740	3306.348	191.502	0.000**
Factor A	2	8437.281	4218.640	244.342	0.000**
Factor B	1	346.363	346.363	20.061	0.000**
A X B	2	7748.097	3874.048	224.383	0.000**
Error	20	345.307	17.265	-	-
Total	29	-	-	-	-

Coefficient of Variation = 9.185

Critical Difference Values

-	CD 5%	CD 1%
Factor A	3.876	5.287
Factor B	3.165	4.317
Treatments/(A x B)	5.482	7.477

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

respectively. When exposed for 48 h in *An. stephensi* 61.66% mortality resulted and 10% mortality was observed in *Ae. aegypti* (Fig. 27.B). However a higher dose of 7.5 µl/ml caused 30% mortality in *Ae. aegypti* larvae on 24 h exposure and no further increase was observed in mortality on 48 h exposure.

The difference in mortalities in *An. stephensi* larvae exposed to *P. citrinum* metabolites obtained from 20 d culture was not significant between the 3 replicates/dose ($F=0.953$, $p=0.409$), it was highly significant between the 4 doses ($F=8.031$, $p<0.01$). The effect of time on mortality was also significant ($F=5.514$, $p<0.05$) (Table 17).

82.5% mortality was observed in *Cx. quinquefasciatus* 3rd instar larvae, on 24 h exposure to *P. citrinum* metabolites from 20-day old culture at a dose of 5 µl/ml, whereas on 48 h exposure 100% mortality was achieved (Fig. 27.C).

The larvae exposed to methanol fraction were sluggish and a complete cessation of feeding was observed. The control larvae were active and consumed the feed normally.

For the large scale extraction of metabolites 14-day old culture of *P. citrinum* was used as they were more lethal than those extracted from 20-d-old culture.

4.7 Larvicidal activity of partially purified metabolites:

P. citrinum cultured for 14 days on large scale gave a mycelial mat of 288.6 gm. Methanol extract of 850 ml obtained from this was concentrated to give a final volume of 156 ml. This was refrigerated until further use. This extract at a dose of 10 µl/ml i.e. at 1% concentration resulted in 65% mortality in *An. stephensi* and 83.3% in *Cx. quinquefasciatus* 3rd instar larvae, on 24 h exposure and 94.1% mortality in *An. stephensi* and 100% in *Cx. quinquefasciatus* larvae on 48h exposure. In the bioassay

carried against *An. stephensi* 3rd instar larvae the LC_{50} value of metabolites extracted from 14 d culture of *P. citrinum* was 4.429 µl/ml on 24 h exposure.

The methanol extract was further separated and partially purified fractions of metabolites were tested for larvicidal activity against the larvae of *Cx. quinquefasciatus* which was the most susceptible species at a dose of 10 µl/ml. It was observed that the Pet ether fraction showed high larvicidal activity against *Cx. quinquefasciatus* as mortality observed after 24 and 48 h exposure was 93% and 98% respectively. The chloroform fraction caused 38.3% and 43% mortality on exposure of 24 and 48 h respectively. The ethyl acetate fractions caused mortality of 6.6% on 24 h exposure which did not increase with increase in exposure time. The n-butanol and aqueous fractions showed the lowest activity with mortality of 1.6% in both which did not change with increase in exposure time (Fig. 28).

In the larvae exposed to Pet ether fraction peculiar aggregation behaviour was observed. Further sluggishness and curling of the body was also observed. Except for the larvae exposed to aqueous fraction, in all the other fractions, feed was not consumed by the experimental larvae. In the larvae exposed to chloroform fraction, wriggling and curling movements showing irritability was observed also they showed slow movements compared to the control larvae. The larvae exposed to ethyl acetate, n-butanol and aqueous fractions were active similar to the control. The control larvae were active and consumed the feed.

4.8 Analysis of active fraction by TLC, NMR and IR spectroscopy:

The TLC of the methanol, pet ether and chloroform fractions on silica gel plates gave spots in all the three fractions when subjected to iodine chamber (Fig. 29). In the first solvent i.e. 30% ethyl acetate in pet ether the methanol fraction seperated to give 2

Table 16: Analysis of variance values for bioassay of *P. citrinum* metabolites extracted from 14 d culture vs. *An. stephensi* 3rd instar larvae exposed for 24 and 48 hours.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Replications	2	75.315	37.657	0.371	0.699
Treatments	5	1169.380	233.876	2.302	0.123
Factor A	2	583.534	291.767	2.872	0.103
Factor B	1	539.162	539.162	5.306	0.044*
A X B	2	46.684	23.342	0.230	0.799
Error	10	1016.077	101.608	-	-
Total	17	-	-	-	-

Coefficient of Variation = 18.229

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

Table 17: ANOVA values for bioassay of *P. citrinum* metabolites extracted from 20 d culture vs. *An. stephensi* 3rd instar larvae exposed for 24 and 48 h.

Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Replications	2	142.079	71.039	0.953	0.409
Treatments	7	2323.662	331.952	4.452	0.008
Factor A	3	1796.365	598.788	8.031	0.002**
Factor B	1	411.114	411.114	5.514	0.034*
A X B	3	116.183	38.728	0.519	0.676
Error	14	1043.854	74.561	-	-
Total	23	-	-	-	-

Coefficient of Variation = 21.464

Factor A = Dose; Factor B =Time; F cal = F calculated value; F prob = F probability; * = significant, ** = highly significant.

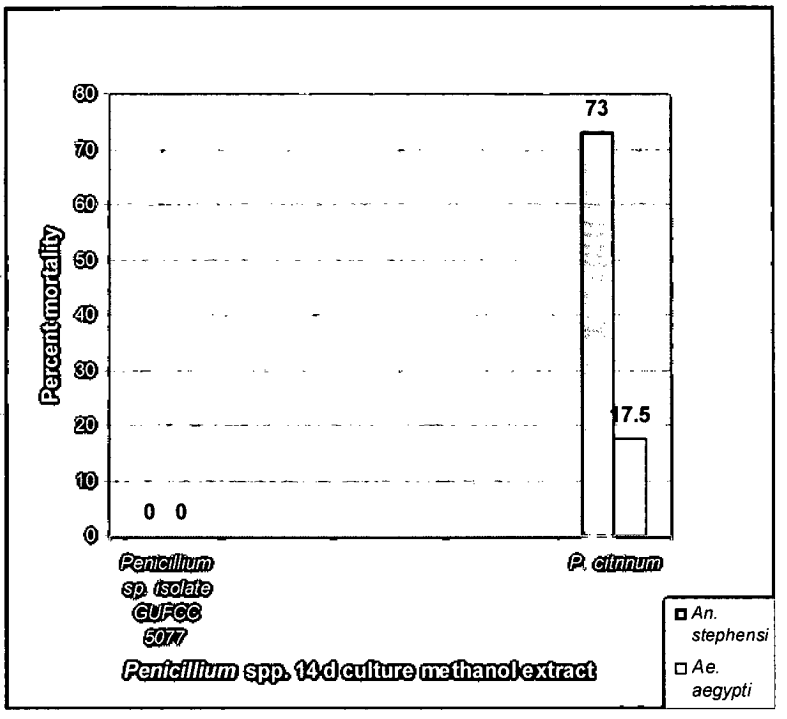


Fig. 27. A Larvicidal bioassay of *Penicillium* spp. metabolites in methanol extract from14-d-old culture against 3rd instar larvae of mosquito vector spp.

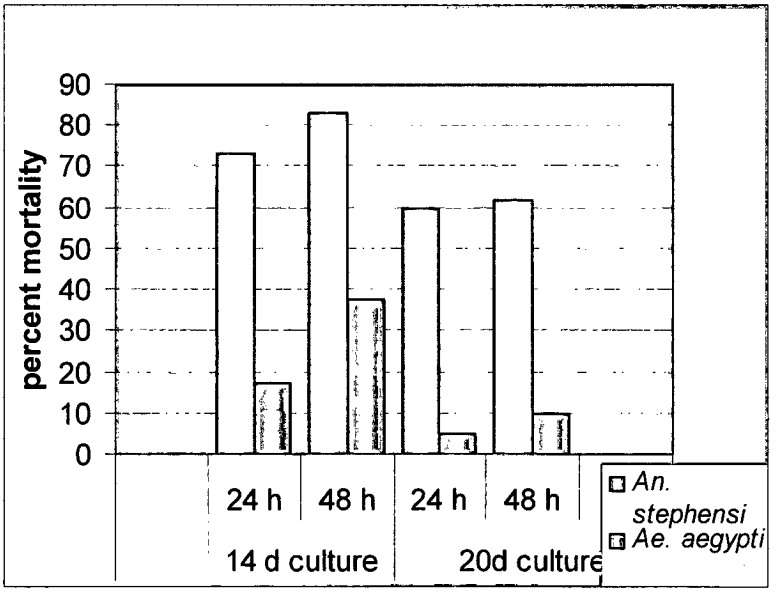


Fig. 27. B Larvicidal activity of *P. citrinum* metabolites in methanol extracts against 3rd instar larvae of mosquito vector spp.

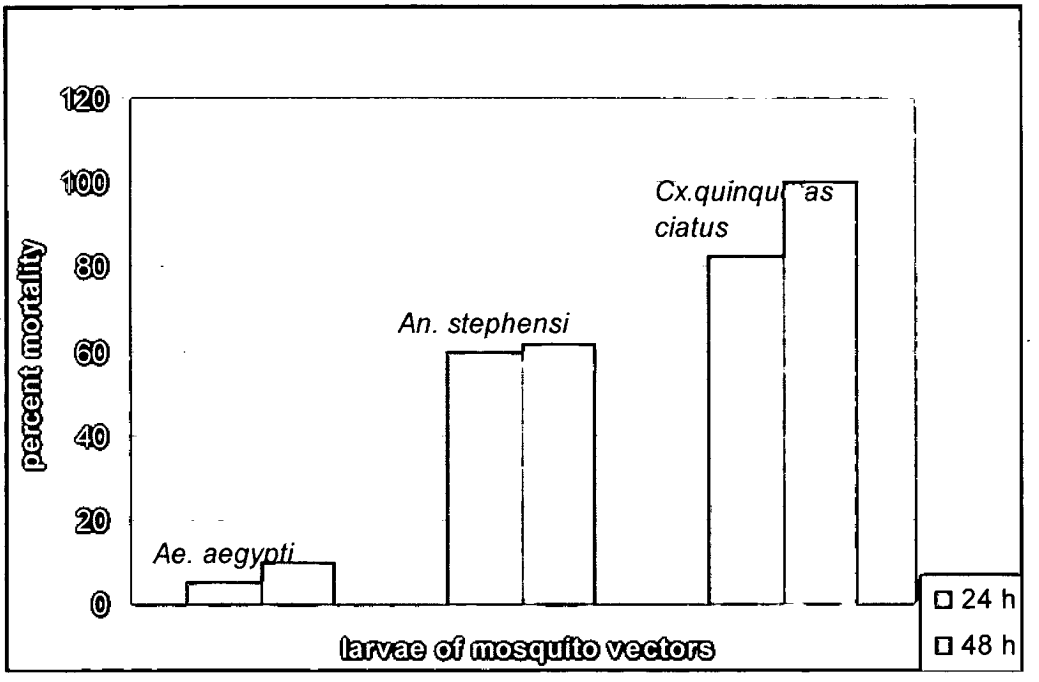


Fig. 27. C Larvicidal activity of *P. citrinum* metabolites from 20-d-old culture in methanol extract against 3rd instar larvae of mosquito vector spp.

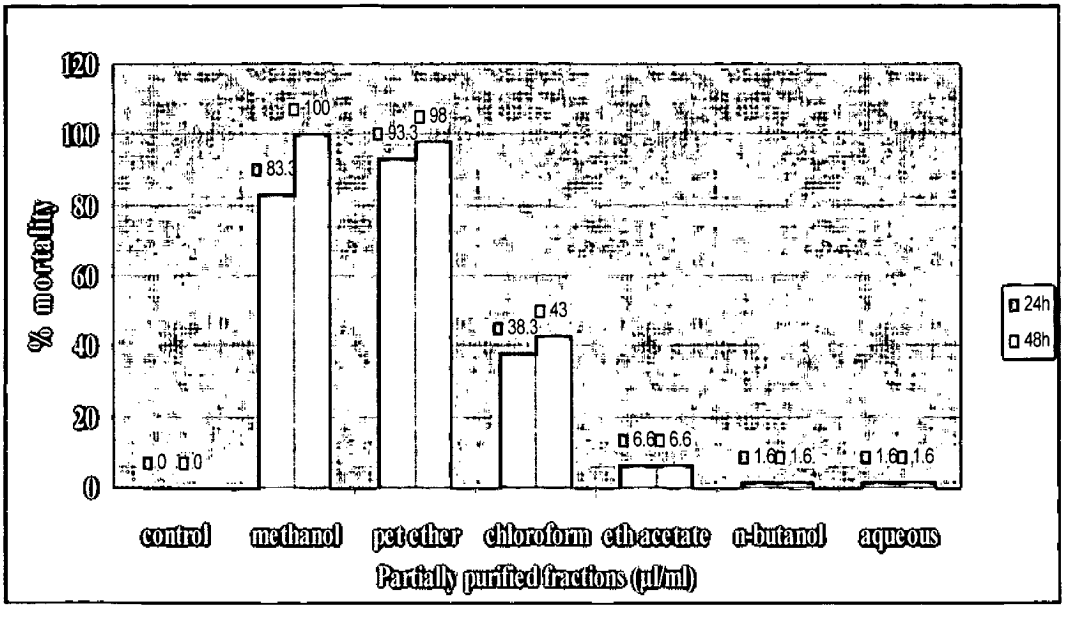


Fig. 28. Results of larvicidal bioassay of partially purified metabolites of *P. citrinum* against *Cx. quinquefasciatus* 3rd instar larvae.

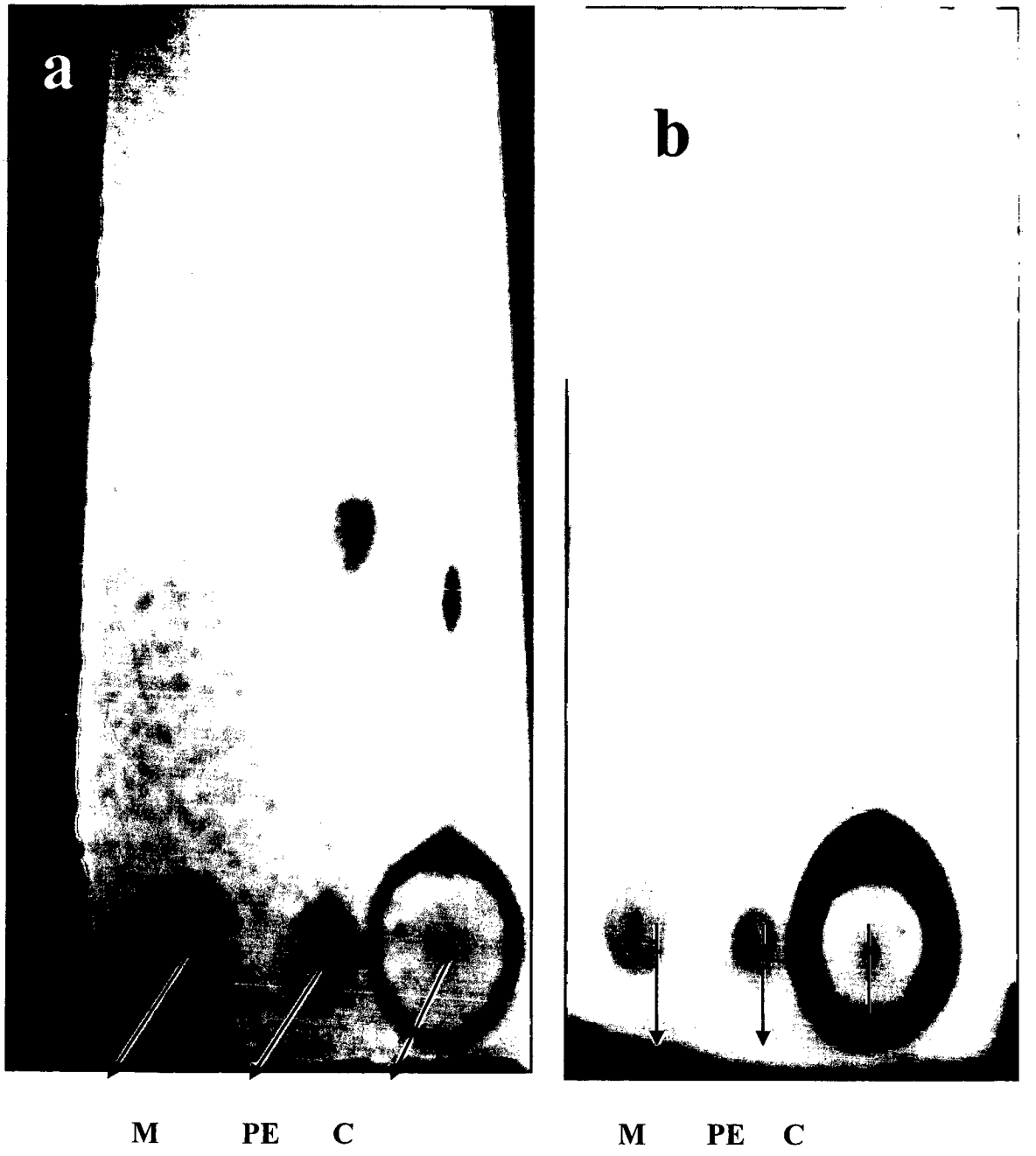


Fig. 29 Thin Layer Chromatography of Methanol (M), Pet ether (PE) and Chloroform (C) fractions of *P. citrinum* crude extracts using two different solvent fronts **a.** 30% ethyl acetate in Pet Ether **b.** 25% ethyl acetate in Pet Ether

spots with Rf values 0.67 and 0.76, the pet ether fraction seperated to give 3 spots with Rf values 0.52, 0.69 and 0.77, the chloroform fraction seperated to give 2 spots with Rf values 0.33 and 0.59. In the second solvent i.e. 25 % ethyl acetate in pet ether the methanol fraction seperated to give 2 spots with Rf values 0.48 and 0.59, the pet ether fraction seperated to give 2 spots with Rf values 0.41 and 0.52, the chloroform fraction seperated to give 2 spots with Rf values 0. 22 and 0.35. The relative Rf values in the fist solvent show that the compounds in the chloroform fraction have high polarity as their Rf values were low, with the compounds in the pet ether fraction having lower polarity and the compounds in the methanol fraction having the lowest polarity. The results of TLC indicated the active fractions are made up of 2-3 compounds.

From the NMR of Pet ether fraction based on the spectral pattern in between δ 1-2.5 ppm the active compound seemed to be fatty acid (Fig. 30.A). The Pet ether fraction on further processing gave two layers an organic layer and an aqueous layer. The two layers were subjected to NMR. When the spectral patterns of these two layers were compared with the spectral pattern of the initial unprocessed Pet ether NMR, the organic layer NMR (Fig. 30.C) looked similar to the Pet ether NMR. Therefore the active compound was in the organic layer. If the active compound was an acid, it would have been extracted in the aqueous layer. But the NMR spectra of the aqueous layer (Fig. 30.B) did not match with the initial NMR of Pet ether fraction. Hence the active compound was not an acid.

This was supported by the data from IR spectra of the organic layer (Fig. 31.A) and aqueous layer (Fig. 31.B). The C=O stretching band observed in the latter at 1712 cm^{-1} and the absence of a prominent –OH band at 3300-3600 cm^{-1} indicated that the compound is not an acid. Usually in Pet ether fraction fatty acids are obtained. After

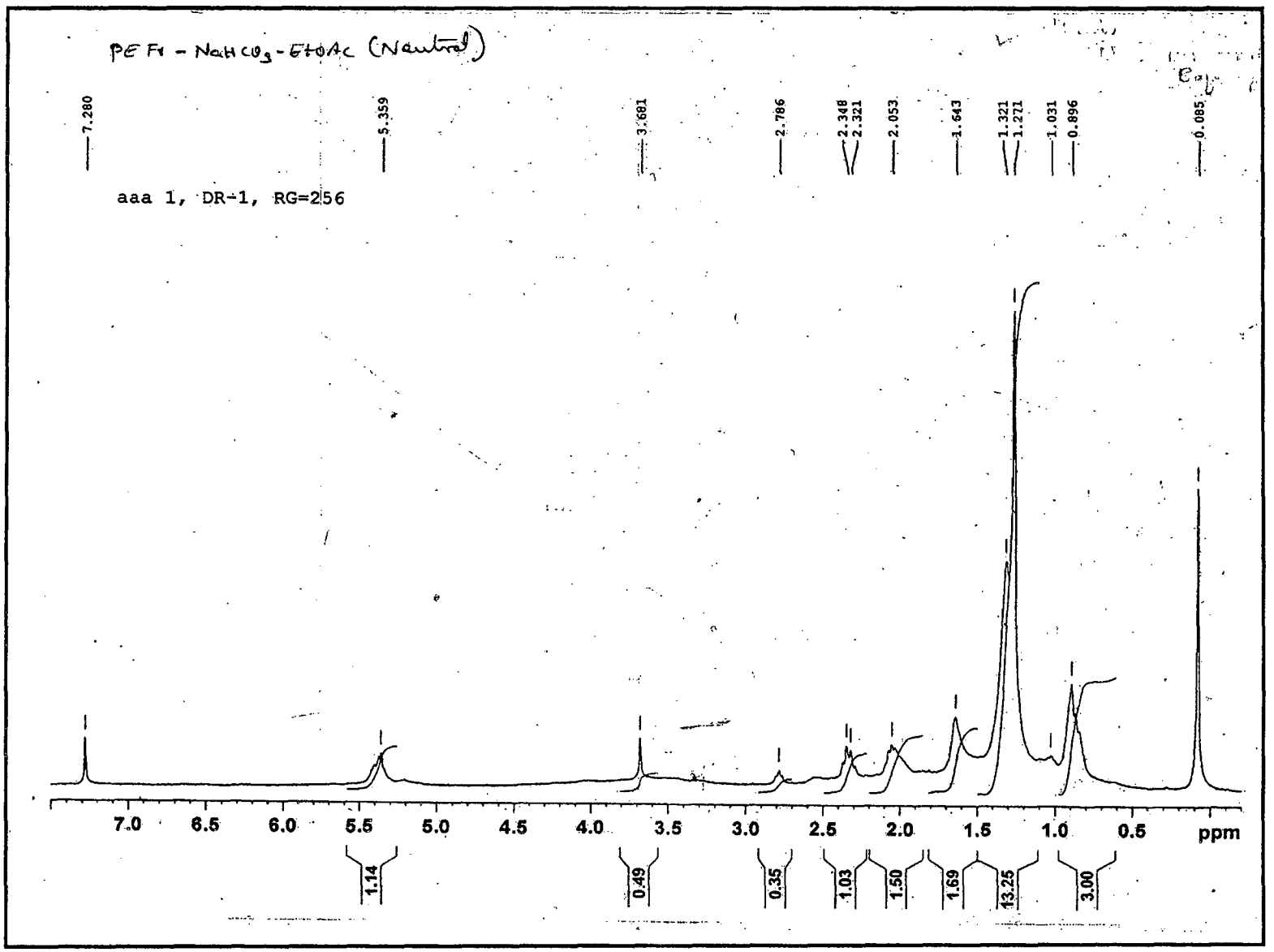


Fig. 30. A NMR of Pet ether fraction

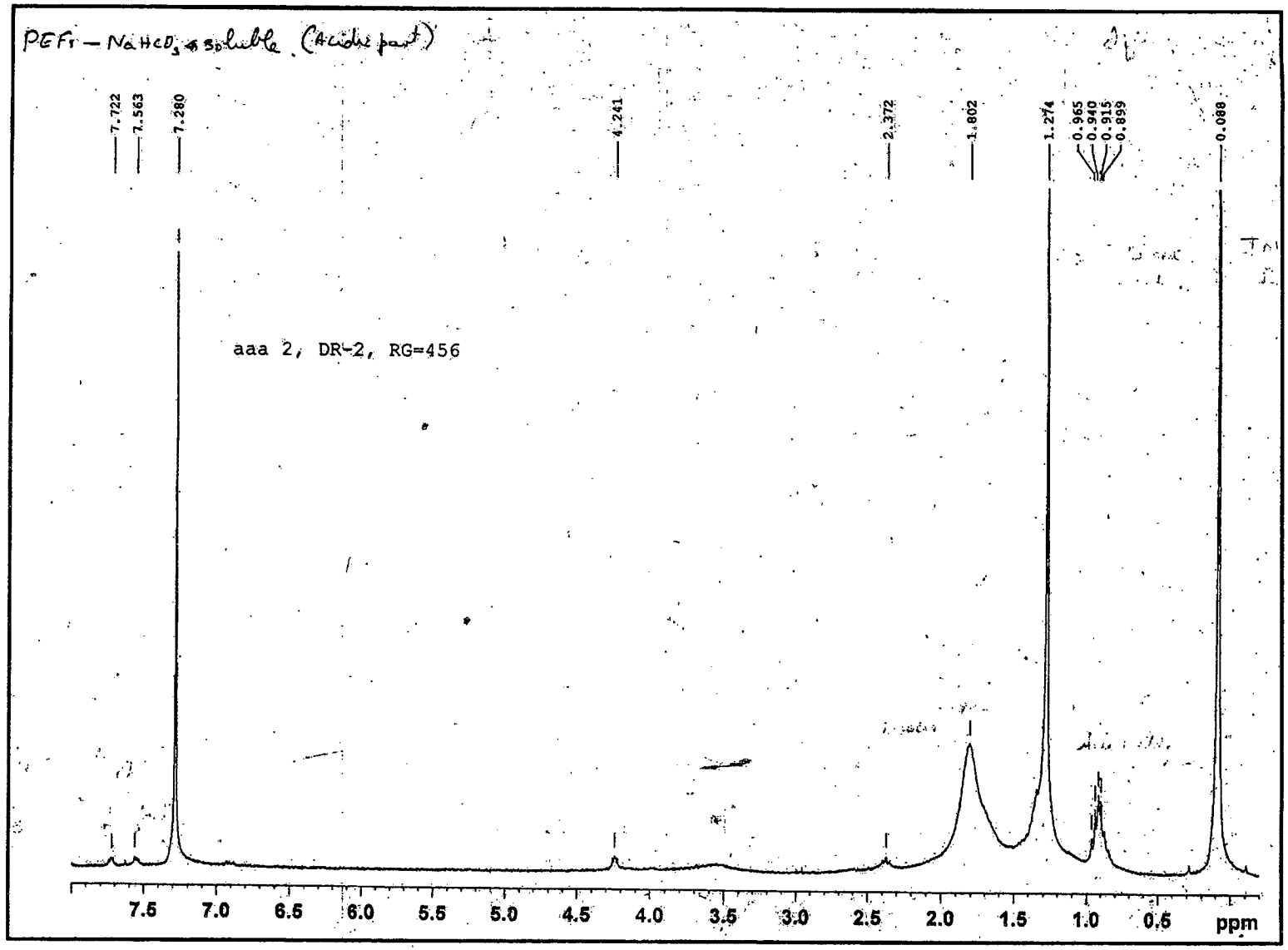


Fig. 30.B NMR spectra of the aqueous layer of processed Pet ether fraction

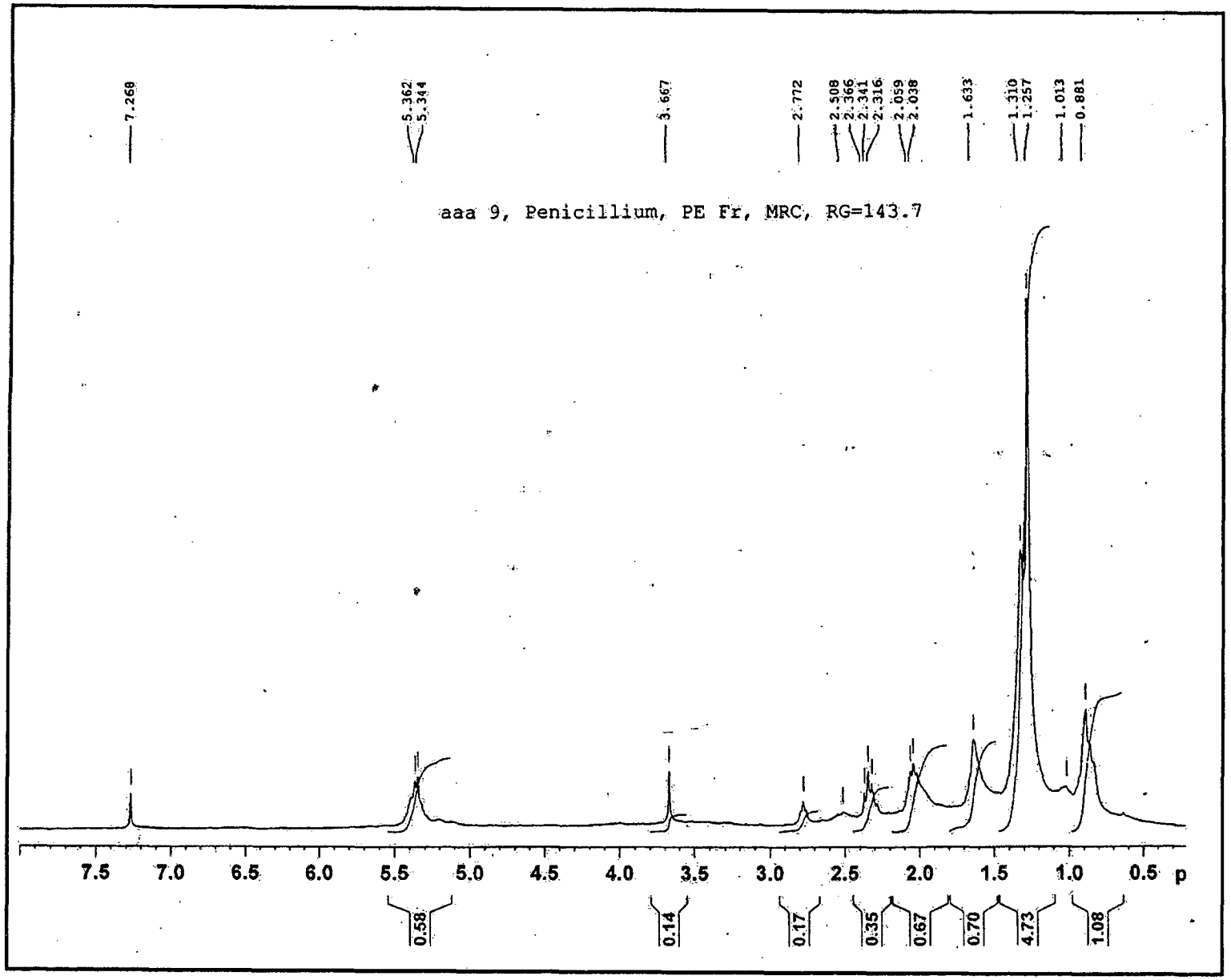


Fig. 30.C NMR spectra of the organic layer of processed Pet ether fraction

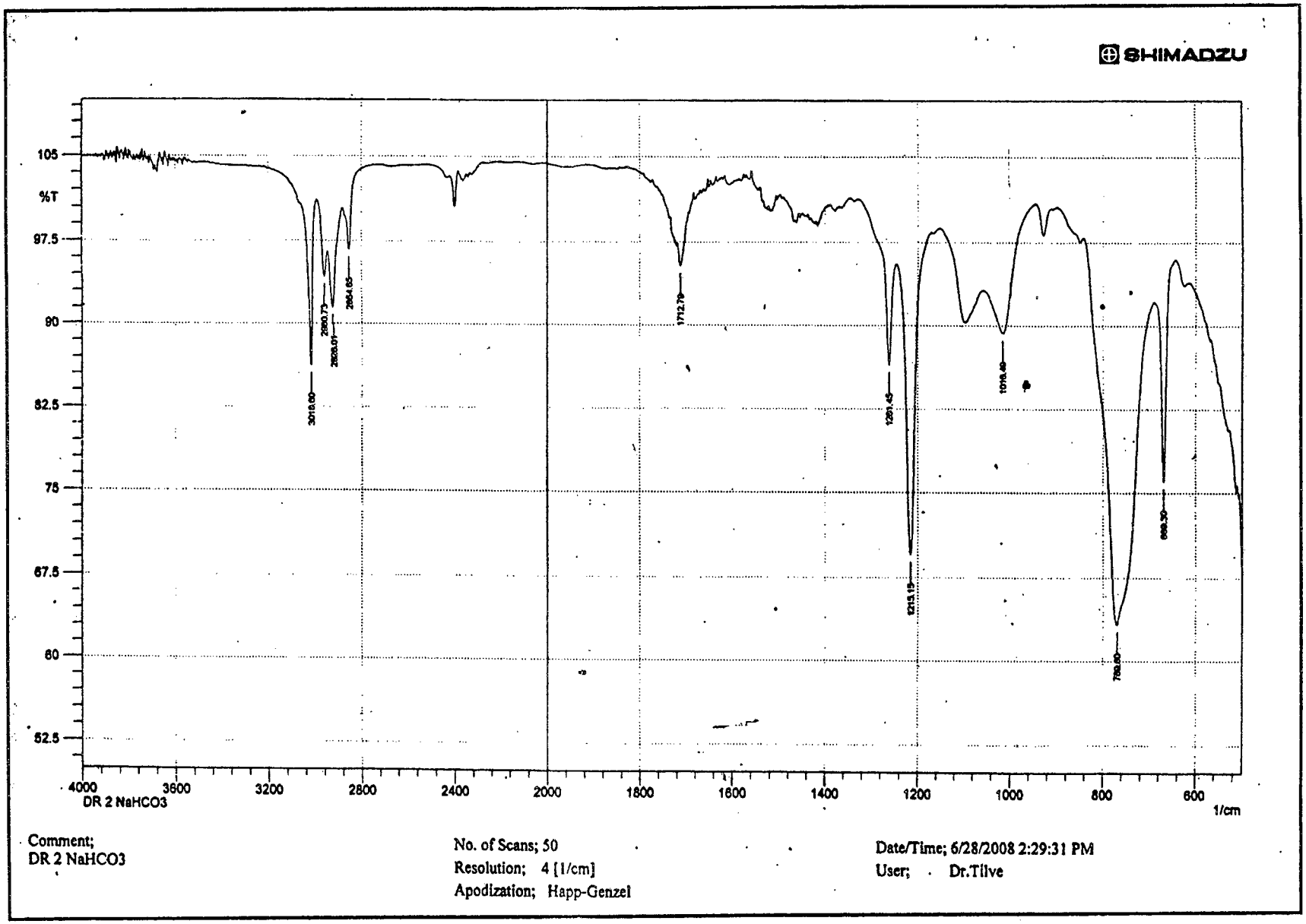


Fig. 31.B IR spectra of the aqueous layer of processed Pet ether fraction

ruling out the probability of an acid as the active compound and looking at the spectral pattern of the initial NMR of Pet ether fraction and the NMR of organic layer of the processed fraction between δ 3.5-4.0 ppm the active compound might be a methyl ester.

4.9 Production and estimation of enzymes of active fungal isolates:

4.9.1 Protease assay:

Time course for the production of protease by *P. citrinum* was studied in shake flasks. The profile of protease production was followed by conducting the fermentation for a period of 336 h i.e. 14 days. Fig. 32 shows that the protease activity could be observed to reach maximum activity starting from 48 h of fermentation the activity being 0.19 U ml^{-1} min^{-1} and reached the peak of activity in the region of 48 h to 168 h i.e.7^{th} day of fermentation and then reduction in the protease activity was observed at 264 h (2.49 U ml^{-1} min^{-1}) and the activity was almost steady at 336 h (2.64 U ml^{-1} min^{-1}). Highest protease activity of 7.78 U ml^{-1} min^{-1} was obtained after 168 h of incubation. *G. roseum* isolate GUFCC 5039 showed protease activity on incubation in shake flask culture. At 168 h of incubation, it showed an activity of 4.82 U ml^{-1} min^{-1} which was less than that exhibited by *P. citrinum*.

4.9.2 Chitinase assay:

P. citrinum showed production of extra cellular chitinase in shake culture at room temperature. The enzyme production was observed at 24 h of incubation and it showed an increase with passage of time. Highest enzyme activity of 0.012 U ml^{-1}

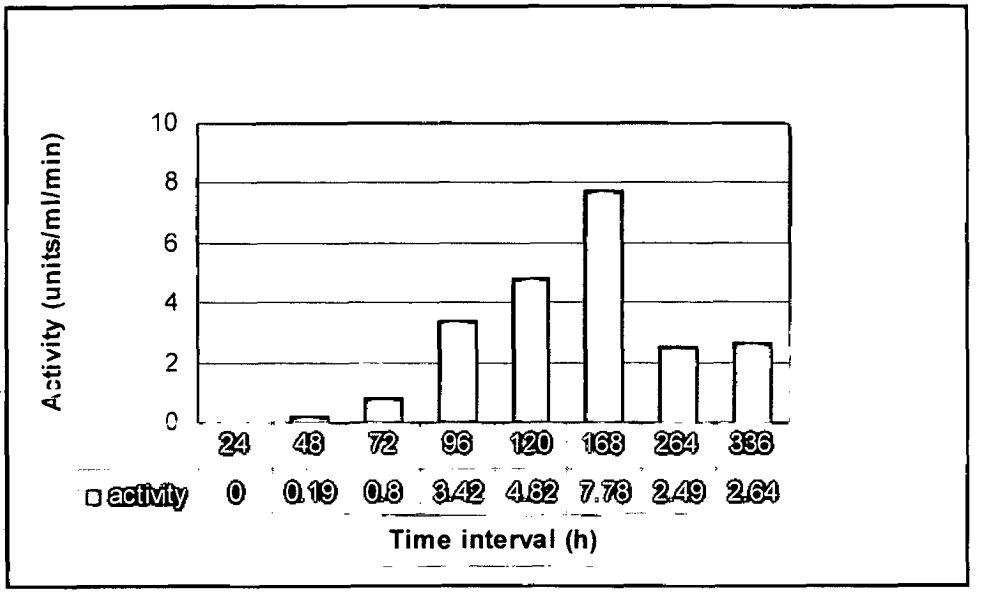


Fig. 32 Protease activity of *P. citrinum*

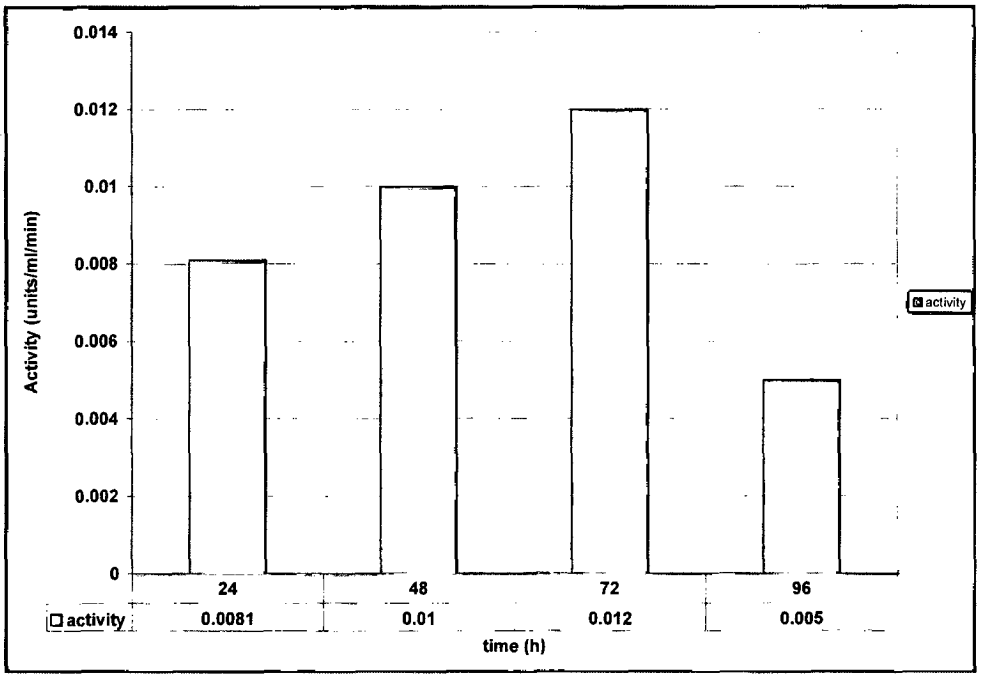


Fig. 33 Chitinase activity of *P. citrinum*

min^{-1} was obtained after 72 h of incubation and thereafter a decrease at 96 h was observed with activity reducing to 0.005 U ml^{-1} min^{-1} (Fig. 33).

4.10 Evaluation of bio-safety of *P. citrinum* to non-target organism:

4.10.1 Vertebrate bio-safety testing:

The larvivorous fishes *Aplocheilus blocki* were exposed for 7 days to *P. citrinum* at a conidial dose of 34.76 x 10^7 spores/ml, which was 4.34 folds higher than the LD_{50} of *P. citrinum* (80.13 x 10^6 spores/ml) against 3rd instar larvae of *Ae. aegypti*. The following morphological and anatomical observations were made in the exposed fishes: Ventral side of the head region turned pinkish in colour (Fig. 34.D). The base of dorsal fin showed black pigmentation in the form of small spots (Fig. 34.C). In the gill chamber green bolus-like mucilagenous mass was observed (Fig. 34.E) which on plating on malt extract agar showed growth of *P. citrinum*. Overall 9% mortality was recorded in the test fishes. No fungal growth was noticed in the eyes, operculum, pectoral, pelvic, ventral and caudal fins. Skin, scales, muscles and gills appeared normal as in control fishes. Alimentary canal had no apparent effect. Gall bladder and liver appeared normal with no abcessions, septicemia, lesions, tenderness or fluffiness (Fig. 34.F). The control fishes did not show any change and no mortality was recorded in them (Fig. 34.A, B).

4.10.2 Invertebrate bio-safety testing:

The invertebrate non-target heteropteran insect used to study the bio-safety of the effective fungus was identified as *Limnogonus* (*Limnogonus*) *fossarum fossarum* (Fabricius, 1775).

L. fossarum fossarum were exposed to conidial suspension of *P. citrinum* for 4 days and observed for 10 days for sluggishness, exogenous growth of fungus on the body

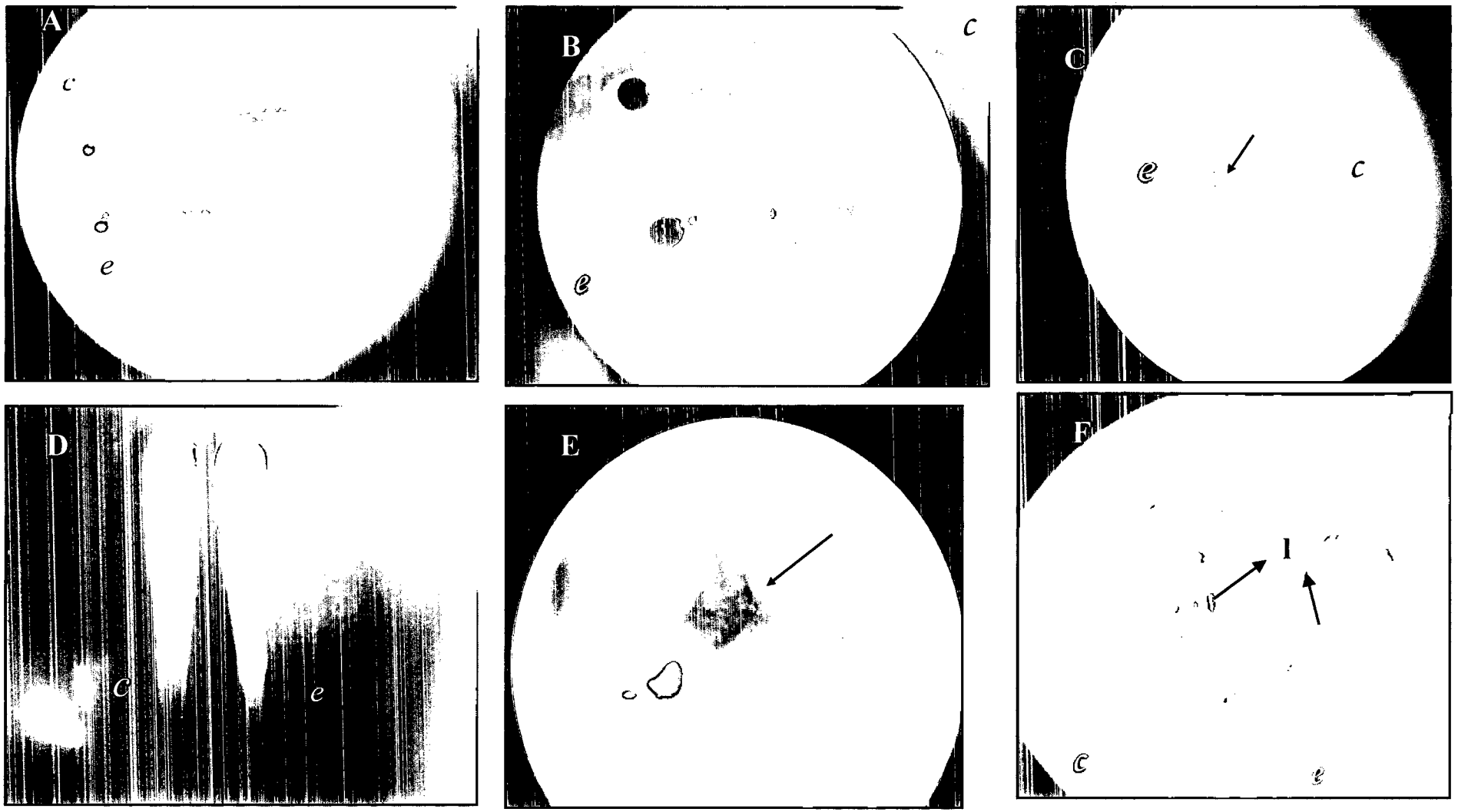


Fig. 34 A-F *Aplocheilus blocki* (control *c*) and (experimental *e*) fish exposed to *P. citrinum.* A. Lateral view control (above) experimental (below) B. head and trunk. C. Exposed fish on left, arrow shows dorsal fin pigmentation and control on right D. Ventral view of control and experimental. E. Gills- green bolus in experimental. F. Alimentary canal exposed control left experimental right, no abcessions or fluffiness or tenderness in liver, l=liver.

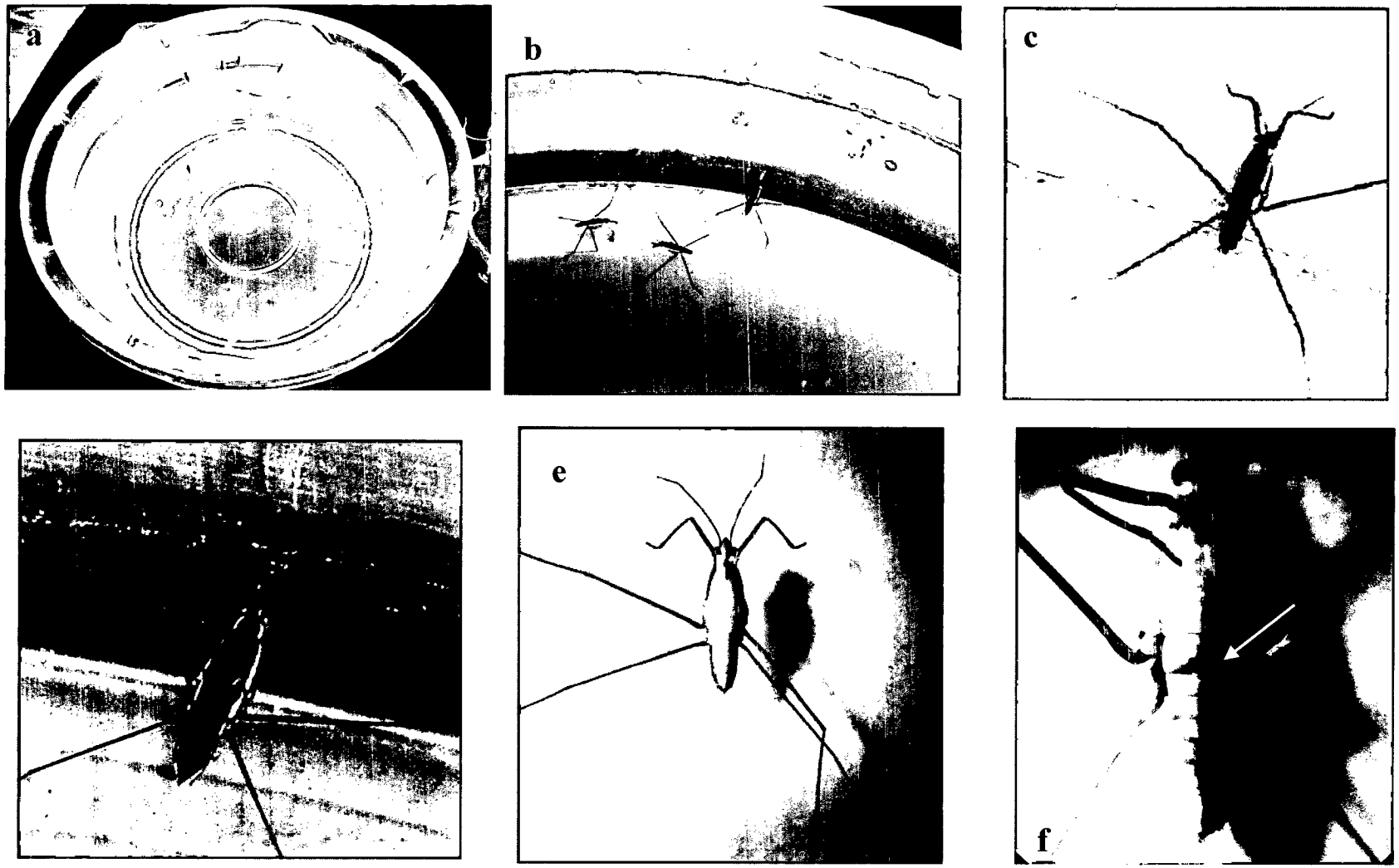


Fig. 35 ***Limnogonus (Limnogonus) fossarum fossarum*** **exposed to** ***Penicillium citrinum*** **a. Control b. Experimental exposed to conidial suspension c. Dorsal surface of control d. Dorsal surface of experimental e. Ventral surface of control f. Secretion of scent gland in the experimental.**

surface and mortality. All exposed *L. fossarum fossarum* were found to be active. No growth of the fungus was observed on body surface on the lateral, ventral and dorsal surfaces (Fig. 35.a-f). In the exposed insects the metathoracic scent glands were active (Fig. 35.f). A low grade mortality of 4% (1/25) was observed in the experimental insects.

The non-target insect *L. fossarum fossarum* exposed to metabolites of *P. citrinum* extracted in methanol at a dose of 40 µl/ml to study their bio-safety did not show any sluggishness. No mortality was observed in the exposed insects. The metabolites of *P. citrinum* extracted in methanol appeared to be biologically safe.

Chapter 5

DISCUSSION

5.1 Sourcing of entomopathogenic fungi and taxonomy:

In the present study it was planned to have an insight into the sourcing of entomopathogenic fungi from nature. Entomopathogenic fungi can be found infecting living as well as dead insects in a variety of habitats. So an exercise was carried out to collect samples from aquatic and terrestrial habitats, laboratory colonies of mosquitoes and agro-ecosystems. As shown in Table 7 entomopathogenic fungi could be sourced from different localities of Goa. The easy availability of these fungi indicates that Goa being humid with tropical climate has rich bio-diversity of entomopathogenic fungi. An earlier solitary effort in this direction had yielded a rich haul of 40 mosquito-pathogenic fungal isolates from this region (Keshava Prasad *et al.*, 2000). There exists definitely a great scope to tap this potential further from this and similar regions in the country. In the present study, we have deployed different techniques for the recognition of potential sources and isolation of mosquitopathogenic fungi. For example, the diseased insects were recognized on the basis of changes in their natural colouration, visible growth of the fungus on the outside of their cadavers, melanized areas on the cuticle, fragility, hardening of the cuticle, peculiar behaviour like lack of feed intake and tremors or curling showing irritability or mummification as has been earlier reported (Lacey & Brooks, 1997).

Sourcing of fungi was also done from Goa University Fungal Culture Collection. Seven fungal isolates which had previously shown promising larvicidal activity (i.e. larval mortality of 50% or above) on screening against 2nd instar larvae of *Cx. quinquefasciatus* were chosen for the present study. They were then screened for their activity against higher instar larvae especially the third instar larvae of the disease vectors viz., *Cx. quinquefasciatus* and *An. stephensi*. We found that the traditional taxonomic tools such as keys based on variations in morphological characters to be

quite handy for the identification of these isolates (Bessey, 1950; Ainsworth *et al.*, 1973; Kendrick, 2000).

5.2 Analysis of larvicidal potential and bio-efficacy of fungal isolates:

Preliminary testing of fungi for larvicidal potential

All the three isolates viz., *Gliocladium*, *Penicillium* and *Trichoderma* were effective in killing 3rd instar *Cx. quinquefasciatus* larvae at a dose range of 10^5-10^6 conidia/ml. In a previous study Keshava prasad *et al.* (2000) had screened 14-day-old culture of these fungi for larvicidal activity against *Cx. quinquefasciatus* 2nd instar larvae using a dose range of 10^4-10^5 conidia/ml. Hence the results of this and the previous study were more or less comparable in this regard. Both 2nd and 3rd instar larvae showed complete susceptibility to *Gliocladium* sp. isolate GUFCC 5044 on 24 h exposure and *P. citrinum* isolate on 48 h exposure at the same dosage implying thereby that these are indeed promising mosquitopathogens which could be suitably formulated and exploited for vector control. Upon repeated sub culturing, some of these isolates tended to loose some virulence. For example, *G. roseum* isolate GUFCC 5040 showed reduced virulence as larval mortality reduced from 100% to 72%, while in case of *Trichoderma* sp. isolate GUFCC 5088 drastic decrease in activity from 100% to 16% was observed in spite of the fact that much higher doses of $6.9x10^6$ spores/ml and $14.19x10^6$ spores/ml respectively were used in our study as compared to an earlier study in which doses between 10^5-10^6 spores/ml were used. On the contrary, in case of *T. atroviride* isolate, a marginal increase in larvicidal activity from 65% to 70% mortality was observed at the same dose which showed that virulence was maintained in this isolate in spite of subculturing. However, this mortality was achieved on 72 h exposure in the present experiment as compared to 120 h exposure in the previous

study indicating that the 3rd instar larvae were more susceptible than the 2nd instar that were used in the previous study. Hence the exposure time to affect mortality was 48 hours shorter than in the previous experiment. In an earlier study Mohanty and Prakash (2002) have also observed that compared to 1st and 2nd instars of *An. stephensi* larvae the 3rd instar were more susceptible when exposed to different conidial concentrations of *Chrysosporium lobatum*. The age of larva seems to be a deciding factor for the virulent activity of the mosquito-pathogenic fungi.

We found that *T. atroviride* conidial suspension was much less effective against *An. stephensi* 3rd instar larvae as it resulted in only 25% mortality in spite of the fact that almost two-fold higher dose was used against *An. stephensi* as compared to *Cx. quinquefasciatus*. This could be attributed to the manner in which the spores attach to the host and also due to host immune system which might be suppressed differently in different species (Chandler *et al.* 1993). Similarly in bioassays conducted by Serit & Yap (1984), the 3rd instar larvae differed in their susceptibilities to *Tolypocladium cylindrosporum* as *Mansonia uniformis* larvae were the most susceptible followed by *Culex quinquefasciatus*, *Anopheles balabacensis* and *Aedes aegypti* in decreasing order. Therefore mosquito-pathogenic fungi seem to be quite selective in their targets and mosquito species show variation in vulnerability.

Our study shows that age of the culture of fungi seems to matter with regard to their pathogenicity to vector species. For example, spore suspension obtained from 21 day old culture at a 1.7 times higher dose (18 x 10^6 spores/ml) of *G. roseum* isolate GUFCC 5040 resulted in similar mortality (47.4%) in *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure while 46.7% mortality was observed on 24 h exposure to 14 day old culture at a dose of (10.52 x 10^6 spores/ml). Thus conidial suspension obtained from 14 d old culture of *G. roseum* had rapid action as compared to 21 d old

culture. The pace of action on target vector is one of the important criteria for adoption of any microbial agent in the vector control programmes.

As the surviving larvae of vector mosquitoes exposed to *G. roseum* did not develop further into pupae and adults did not emerge from them, it appeared that conidia from 21 d had not only larvicidal but also growth inhibitory activity against *Culex* 3rd instar larvae quite similar to insect growth regulator (IGR) compounds. Hence it will be of interest to reconfirm whether this fungus mimics and can be used as an IGR by conducting an in-depth study. Further studies are also warranted on the surviving larvae as they could provide an insight into the sub-lethal effects of this fungus e.g. on fecundity of F_1 adults, fertility, egg laying, etc. in the susceptible vectors. When mature larvae and pupae of *Chilo suppressalis* were treated with sub-lethal doses of *B. bassiana*, the number of eggs laid by the adults formed from them were in an inverse ratio to the spore dosage to which they were exposed (Baye & Doye, 1976). This clearly indicates that fecundity of the next generation of target population exposed earlier to fungi at immature stages is affected and similar phenomenon needs to be explored in detail in mosquitoes exposed to various mosquitopathogenic fungi.

5.3 Mode of invasion of active fungi in mosquito larvae:

Mosquito-pathogenic fungi invade mosquito larvae through cuticle, spiracles or by ingestion depending upon which the development may take place in the gut, head or anal tissues, etc. (Lacey *et al.*, 1988; Sweeney, 1975; Sweeney *et al.*, 1983). Larval death may be attributed to histolysis, mechanical interference with spiracular functions, production of potent mycotoxins or physiological starvation (Ciegler, 1976; Lacey *et al.*, 1988).

In the present study, *Cx. quinquefasciatus* larvae exposed to *Gliocladium* sp. isolate GUFCC 5044 showed profuse mycelial growth on their cuticle. The haemocoel of the larvae was ramified with fungal mycelia and the organ tissues were disrupted at 24 h exposure. Also melanization was seen around invading hyphae in some areas of the midgut. These observations are similar to the ones made by Goettel (1988a) in the pathogenesis of *Tolypocladium cylindrosporum* in *Ae. aegypti* larvae.

When exposed to *T. atroviride,* the larval gut of *Cx. quinquefasciatus* and *An. stephensi* appeared packed with green coloured matter on exposure to green spores. Greenish faecal pellets were observed showing that the spores were ingested as well as egested. In *Anopheles* larvae, the conidia from the faecal pellets plated on antibiotic loaded CMA germinated implying thereby that the viable spores that germinate remained unaffected during their passage through larval gut and they escaped the action of gut enzymes or showed resistance to them. Also since the egested spores are viable, they will ensure recycling of fungus. It was observed by Sweeney (1975) and Sweeney *et al.* (1983) in *Cx. fatigans* larvae that the normal route of infection by *Culicinomyces clavisporus* was the digestive tract. *B. bassiana* (Miranpuri & Khachatourians, 1991) and *M. anisopliae* (Al-Aidroos & Roberts, 1978) infect *Ae. aegypti* larvae through the gut confirming that mosquitoes are very much vulnerable to this mode of attack. Reports on insect mortality due to the toxic activities of large number of ingested but ungerminated conidia in the mosquito larvae are also available (Serit & Yap, 1984).

Our studies on the fate of the conidia of *T. atroviride* after being held for more than two hours in the gut of *Cx. quinquefasciatus* larvae, showed that they were still viable. Prior to the exposure to inoculum, the gut contents were purged with sterile India ink to rule out any effects that the gut flora could have on the viability of the conidia.

These results are in accord with those of Goettel *et al.* (1988b) who concluded that the viability of *Tolypocladium cylindrosporum* spores was not reduced after a 2.5 h passage through the gut of the 2nd instar larvae of *Ae. aegypti*. This provides advantage to the fungal species as far as recycling and residual efficacy is concerned. Whether this passage through the host has negative or positive effect on the virulence of pathogen in the vector needs investigation?

The fate of fungal spores in different larvae after ingestion differs widely as reported by different authors. In the gut of *Cx. quinquefasciatus* larvae apparent digestion of *M. anisopliae* conidia was observed by Lacey *et al.* (1988). Disruption of ingested spores of *T. cylindrosporum* in the gut of *Aedes albopictus* was reported by Ravallec *et al.* (1989).

In the present study, the larvae of *An. stephensi*, *Cx. quinquefasciatus* and *Ae. aegypti* rapidly ingested green conidia of *P. citrinum* and within 2 hours, the larval gut was filled with spores which made the gut appear like a green tube under a microscope. Hence these fungi upon indiscriminate ingestion can begin their rapid lethal action soon after they are ingested. It is assumed that the suitable formulation of these fungi if deployed for vector control will be able to begin killing action on larvae almost instantaneously.

Since the larvae of *An. stephensi* show blackening of middle part of their head and thorax, one can easily observe under dissecting microscope that process of melanization also begins soon after ingestion. Melanization is a process of host cellular defense response to a pathogenic fungus as has been earlier reported (Ferron, 1978). Most of the larvae showed this feature after exposure and eventually the whole body of the larvae became black accompanied by mortality showing thereby that the fungal pathogen evokes similar response in majority of the exposed larvae. However

we observed that a few melanized larvae escaped death. Melanin is a polymer which strengthens the cuticle and barricades the entry of parasites and pathogens through this route (St. Leger *et al.*, 1988b). This protective phenomenon has been previously reported and when pathogenic fungus succeeds in penetrating cuticle, the plasmatocytes which are present in the haemolymph accumulate around the fungus and give rise to melanization. Melanin is toxic and antimicrobial (Ourth & Renis, 1993) in nature by virtue of its capacity to bind proteins (Doering *et al.*, 1999) and inhibit microbial lytic enzymes (Bull, 1970). In *Manduca sexta* larval cuticles on induction to melanize exhibited resistance to fungal penetration for duration of 72 h whereas unmelanized cuticles succumbed after 40 h (St. Leger *et al.*, 1988b). It was stated by Golkar *et al.* (1993) that in the larvae of *An. gambiae*, the germ tube penetration by zoospores of *Lagenidium giganteum* provoked an intense melanization in the cuticle which protected 56% of larvae from death. They also observed variations in the host defense reaction with fast and intense melanization in *An. gambiae* as compared to *Ae. aegypti* and *Cx. pipiens* larvae. In the present study, in *An. stephensi* larvae despite the melanization, the highest dose of *P. citrinum* (20.02 x 10^6 spores/ml) yielded a larval mortality of 84 % in 48 h. The degree of melanization differed in the exposed larvae. We observed that the immune response in *An. stephensi* larvae was faster compared to *Cx. quinquefasciatus* and *Ae. aegypti* larvae. Similar to the earlier studies by Lacey *et al.* (1988), the larval death could be due to physiological starvation and/or by production of mycotoxins by spores in the gut. The recovery of fungus on plating the gut of exposed larva proved that the green mass choking the gut was indeed comprised of ingested spores.

In appearance, finally the dead *Culex* larvae had turned pale and shriveled whereas *Ae. aegypti* larvae became transparent. In *Cx. quinquefasciatus* larvae, extensive

mycosis accompanied by emergence of hyphae through the cuticle was observed from the head, thorax, abdomen, anal siphon traversing the gut and haemocoel was observed which showed that once established fungus could extensively grow in larvae bringing in its wake death of the host. Not all the larvae however showed such luxurious mycelial growth as 50-60% of the dead larvae were without any apparent infection. This suggests that mortality in larvae may precede hyphal growth and proliferation of fungus in the host. Similar observations have been made by Goettel (1988a) in *Ae. aegypti* which were exposed to *T. cylindrosporum*. In that case 10-50% of exposed immatures died without any signs of fungal colonization inside the haemocoel and in a few of the exposed larvae, extensive mycelial growth occurred in the foregut, midgut, the colon or rectum but without any penetration of the host tissues. In the present study, no mycelial growth of *P. citrinum* was observed in the case of dead larvae of *Ae. aegypti* and *An. stephensi*.

The SEM studies confirmed that *Cx. quinquefasciatus* larvae exposed to *P. citrinum* had conidial attachment in abundance on to the surface of respiratory siphon and anal lobes, slightly lesser in the thoracic region and in patches on rest of the body. On the other hand, the SEM of the dissected gut of *Cx. quinquefasciatus* showed abundant conidia of *P. citrinum* and of these, a few were germinating as suggested by appressorial formation thus substantiating that the primary route of invasion and infection was through gut.

In case of exposed *Ae. aegypti* larvae, *P. citrinum* conidia were present sparsely on the surface confirming that mode of action was by internalization rather than exogenous growth in this species too.

Further the confirmation of above phenomenon was provided by the SEM of the faecal pellet of *Ae. aegypti* and *Cx. quinquefasciatus* larvae which clearly showed that

intact egested *P. citrinum* conidia were enveloped in the peritrophic membrane. Galli-Vallerio & Jongh (1906) had observed that the larvae of anopheline and culicine mosquitoes exposed to *Aspergillus niger* and *A. glaucus* produced long tails of type II peritrophic membrane. The midgut of mosquito larvae has type II peritrophic membrane which forms a continuous sleeve (or sleeves) that is always present and thought to protect the midgut epithelium from mechanical injury and pathogens (Lehane, 1997). Abedi & Brown (1961) had reported that the resistant and susceptible strains of *Ae. aegypti* larvae voided a visible peritophic membrane in the faeces using it as a vehicle to excrete DDT and DDE when challenged with the insecticides. The resistant strain produced peritrophic membrane 9 times more than the susceptible strain and contained 3 times more DDE and 5-15 times as much DDT as produced by the susceptible strain.

Soares (1982) observed that in larvae of *Ae. sierrensis*, the route of invasion of *T. cylindrosporum* was through the cuticle and alimentary canal. Similar observations were made in our study on mode of action of *P. citrinum* in *Cx. quinquefasciatus* larvae with SEM showing the presence of conidia on the cuticle and inside of the gut. The gut seems to be the major route of invasion as germinating conidia were also seen in the gut in SEM and mycosis was also observed in some of the larvae with light microscopy. There is probability that toxins secreted by the conidia are also contributing to the mortality as the metabolic extracts of *P. citrinum* showed larvicidal activity.

5.4 Subculturing of the three isolates and loss of activity in case of *Gliocladium* sp. (isolate GUFCC 5044) and *Trichoderma atroviride*:

On successive subculturing in artificial media, fungi are known to lose virulence or undergo a change in the morphological characters. Attenuation of virulence is seen in major taxa of entomogenous fungi (Butt *et al.*, 2006). The decline in virulence occurs at different rates in different strains. *V. lecanii* showed loss of virulence after 2-3 subcultures (Nagaich, 1964) others after 10-12 times of subculturing (Hajek *et al.*, 1990), no decline in virulence on subculturing more than 12 times was reported by many (Hall, 1980; Ignoffo *et al.*, 1982; Vandenberg & Cantone, 2004). Aberrant morphology was reported in *Entomophaga maimaiga* after subculturing 50 times in liquid media (Hajek *et al.*, 1990).

This phenotypic degeneration encompasses a change in colour, growth form and reduced sporulation of the fungus. In our study, as discussed earlier, three promising isolates *Gliocladium* sp. isolate GUFCC 5044, *T. atroviride* and *P. citrinum* were chosen for studying mode of action and their bio-efficacy on mosquito larvae and they were repeatedly subcultured. *Gliocladium* sp. isolate GUFCC 5044 showed change in morphology, *T. atroviride* showed reduction in virulence while *P. citrinum* was the most stable and showed neither morphological change nor loss of virulence over a period of three years of repeated sub culturing in the artificial media. Hence this species was chosen for further evaluation of its bio-efficacy, metabolites, safety status to NTO and enzyme production.

The pathogenicity of *Culicinomyces clavisporus* to mosquitoes did not decrease apparently on repeated subculturing on artificial media (Cooper & Sweeney, 1982) in our study too there was no decline in the pathogenicity of *P. citrinum* on prolonged subculturing on artificial media. On the other hand, after several weeks of storage at

25°C aqueous suspensions of conidia lost their infectivity to mosquito larvae (Sweeney, 1981). In contrast in the present study however, the *P. citrinum* conidial suspension stored in the refrigerator was active after storing for a period of one year. Therefore this species has number of qualities that merit its further scientific exploration for development as mosquitocidal mycoinsecticide.

5.5 Analysis of bio-efficacy of promising isolate:

Out of the three main bioassays carried to assess the bio-efficacy of *Penicillium citrinum* against the 3rd instar larvae of three test vector species, two assays i.e. against *An. stephensi* and *Ae. aegypti* were amenable to probit analysis while the one against *Cx. quinquefasciatus* larvae was not. In a bioassay, differences in batches of insects can be a source of potential variation (Bucher & Morse, 1963; Burges & Thomson, 1971).

Balaraman *et al.* (1979) obtained 68.5 fold lower LD_{50} values (2 x 10^5 spores/ml) in case of *M. anisopliae* against second instar *An. stephensi* larvae compared with LD_{50} values of *P. citrinum* against the 3rd instar larvae observed in the present study. Serit & Yap (1984) reported in 3rd instar larvae of *An. balabacensis* the LD_{50} value of 4.76 x 10^5 spores/ml on 96 h exposure to *T. cylindrosporum*. Although this concentration was 36 fold lower than *P. citrinum* (LD_{50} = 17.13 x 10^6 spores/ml) against *An. stephensi* in the current study yet in the latter case, the LD_{50} was achieved in 24 h and hence produced rapid mortality in larvae which is crucial in vector control operations. Further by increasing spore concentration of *P. citrinum* by 1.2 folds, i.e. 20.02 x 10^6 spores/ml, the mortality was increased to 84% in 48 h. Similarly, *Lagenidium giganteum* at a higher concentration of 3 x 10^6 spores/ml caused above 90% mortality in the 3rd instar larvae of *An. punctipennis* (Kramer, 1990).

In his study on the effect of different isolates of *T. cylindrosporum* against *Ae. aegypti*, second instar larvae, after ten days of continuous exposure, Goettel (1987c) obtained LD_{50} values in the range of 0.7-67.6 x 10^4 conidia/ml. The LD_{50} value obtained in the present study on continuous exposure of *Ae. aegypti* 3rd instar larvae to *P. citrinum*, was comparatively 100-fold higher with LD_{50} value of 66.03 x 10^6 spores/ml at 48 h exposure but the time required to achieve 50% mortality was 5 times lesser with this species of fungus. Hence higher concentration of conidia can offset the cost of prolonged exposure which is crucial in tropical conditions of high temperature and shorter developmental span of mosquito immatures. da Costa *et al.* (1998) have reported mortality rates from 0 to 6.6% when *Penicillium corylophilum*, *P. fellutanum*, *P. implicatum*, *P. janthinellum*, *P. viridicatum* and *P. waksmanii* species were tested on second instar *Ae. aegypti* larvae and at different concentrations. In comparison, in the present study, the third instar larvae of *Ae. aegypti* showed a higher degree of susceptibility to *P. citrinum* with average percent mortality of 48%, 61.33% and 62.7% on 24 h, 48 h and 72 h exposure respectively.

In our study on 48 h exposure to *P. citrinum* conidial suspensions, the *Cx. quinquefasciatus* larvae were susceptible showing 88% mortality at a dose of 10 x 10^6 spores/ml. However in natural habitats, the spores will quickly settle down out of the feeding zone to less accessible substrata (Lacey *et al.*, 1988). But with proper formulation the spore suspension can be used effectively in the field. Oil formulations diminish effect of UV radiation on spores and are effective in increasing spore survival duration and fungal efficacy against insects (Hong *et al.* 2005, Inyang *et al.* 2000).

The two factor ANOVA also showed that the difference in mortalities was highly significant between different doses in case of *An. stephensi* larvae and *Ae. aegypti*

larvae while it was not significant in case of *Cx. quinquefasciatus* larvae. The effect of time was significant in case of *An. stephensi* while highly significant in case of *Ae. aegypti* larvae and not significant in *Cx. quinquefasciatus* larvae. *Cx. quinquefasciatus* larvae were highly susceptible at very low doses compared to *An. stephensi* and *Ae. aegypti*, the latter requiring a much higher dose. Hence prolonged continuous exposure for a period of 72 h caused a greater mortality in *Ae.* aegypti compared to the other two.

5.6 Assessment of metabolites:

In their studies Vijayan & Balaraman (1991) obtained the LC_{50} values of fungal metabolites of 17 species in the range of 3-24 µl/ml against *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure, whereas in the present study, LC_{50} value was achieved on 24 h exposure with *Trichoderma atroviride* at a slightly higher dose of 26.36 µl/ml. The LC_{50} value of *Chrysosporium tropicum* evaluated against 3rd instar *Cx. quinquefasciatus* larvae was 79 µl/ml (Priyanka & Prakash, 2003), which is three-fold higher than *T. atroviride* observed in the present study. A crude extract of tolypin caused 100% mortality in the larvae of *Cx. pipiens* and *An. maculipennis* at a concentration of 100 µl/ml (Weiser & Matha, 1988b).

Penicillium species seem to be a never ending source of novel bioactive metabolites world-wide (Larsen *et al.*, 2007; Ge *et al.*, 2008; Takahashi & Lucas, 2008). They produce diverse secondary metabolites ranging from acetyl cholinesterase inhibitors (Kim *et al.*, 2001), immunosuppressants, cholesterol-lowering compounds (Kwon *et al.* 2002), mycotoxins (Frisvad & Samson, 2004), antibacterial (Lucas *et al.* 2007) and antifungal (Nicoletti *et al.*, 2007) agents.

In the present analysis *P. citrinum* metabolites extracted in methanol were highly larvicidal with 100% mortality in *Cx. quinquefasciatus* on 48 h exposure while *An. stephensi* larvae were slightly less susceptible with 94.1% mortality. Hence *Cx. quinquefasciatus* larvae were chosen as standard species to test activity of separated fractions. The LC_{50} value of metabolites extracted from 14 d culture of *P. citrinum* was 4.429 µl/ml against *An. stephensi* 3rd instar larvae on 24 h exposure which indicates the extract is not only highly effective but also the mortality can be achieved in short time. The results of bioassays with metabolites from submerged cultures of *T. atroviride* and *P. citrinum* clearly suggest the production of larvicidal toxins. It is thus likely that the larval mortality observed during our studies, in the absence of fungal hyphae, could actually be due to a similar toxin/s produced by ingested conidia in the gut.

It was interesting to observe that *P. citrinum* metabolites extracted in methanol from 14 d culture caused 73% mortality in *An. stephensi* while the metabolites extracted from 20 d culture resulted in 60% mortality. The age of the conidia therefore seems to play an important role in the effectiveness of toxic metabolites. Hence for the separation of active fractions of *P. citrinum* metabolites 14 d culture would be of greater value. Our study has shown that all the three test vector species were susceptible to *P. citrinum* metabolites in the increasing order of *Ae. aegypti*→ *An. stephensi* → *Cx. quinquefasciatus*. *Ae. aegypti* larvae showed least susceptibility requiring much higher doses and exposure time was not significant whereas in *An. stephensi* and *Cx. quinquefasciatus* larvae were far more susceptible and the duration of exposure was significant. The effectiveness of metabolites of *P. citrinum* did not diminish much after storage in the refrigerator for a period of thirteen months as

bioassays carried out with methanol extracts against *Cx. quinquefasciatus* showed a marginal reduction in mortality from 83.3% to 73%.

5.7 Larvicidal activity of partially purified metabolites:

Before running the bioassays to test the activity of partially purified metabolites, an assay was carried out by us to assess the effect of different solvents on the control larvae. The solvents Ethyl acetate, Pet ether, chloroform and n-butanol caused mortality in the *Cx. quinquefasciatus* larvae exposed but no mortality was seen with methanol. Hence we dried each separated fraction and then re-dissolved them in methanol or DMSO. The ethyl acetate and chloroform fractions which could not be redissolved in methanol were dissolved in DMSO. No mortality in *Cx. quinquefasciatus* larvae with DMSO was observed as was expected because DMSO is an inert material. The yellow amorphous granular concentrate of Pet ether fraction was re-dissolved in methanol because its solubility was good.

Bioassay of dichloromethane extract from mycelium of *B. bassiana* at 100 ppm showed activity against *Ae. aegypti* larvae (Gupta *et al.*, 1995). The extract had Beauvericin and two analogues (Beauvericin A and B). Grove & Pople (1980) observed 86% mortality with beauvericin in *Ae. aegypti* larvae after 48 h exposure at 20 g ml^{-1}, but only 39% when half the dose was used (10 g ml^{-1}). With LD_{50} values of 10 to 100 ppm a mixture of 70% destruxin A and 30% B from *M. anisopliae* was shown to be toxic to mosquito larvae (Roberts, 1974).

In a study by Abe *et al.* (2005), the butanol extract from 12-day solid state fermentation of *Penicillium citrinum* F 1539 exhibited insecticidal activity against adult Green peach aphids (*Myzus persicae*). They observed 100% mortality at 1000 ppm on 4-day exposure. In the present study the Pet ether fraction of methanol extract of *P. citrinum* GUFCC 5072 from 14-day submerged culture showed 98% mortality

in *Cx. quinquefasciatus* 3rd instar larvae on 2-day exposure at 730 ppm i.e. at a dose of 0.73 mg ml^{-1}. Comparatively these doses are much lower and hence highly potent. The highly larvicidal Pet ether fraction was of great interest and an attempt was made to further analyse the active fraction. Also it was observed that the chloroform fraction caused 38.3% and 43% mortality on exposure of 24 and 48 h respectively at a dose of 0.87 mg ml^{-1}. As the mortality was below 50%, it was not investigated further. But as the surviving larvae exposed to this fraction did not pupate, we suspected that this fraction has growth inhibitory activity similar to Insect growth regulators which warrants further investigation.

In Russia from the culture broth of *P. citrinum* VKM FW-800 quinocitrinines A and B were obtained which demonstrated antifungal, antibacterial and antiproliferative activities (Kozlovsky *et al.*, 2003). Abe *et al.* (2005) isolated a new tetracyclic quinolone compound quinolactacide from the butanol extract of *P. citrinum* which showed 88% insecticidal activity at 250 ppm against aphids. They have attributed the lower mortality with pure compound to the poor solubility in methanol that was used to dissolve the sample for the bioassay.

The purification of the active fractions carried by TLC in the present work revealed multiple spots using 30% ethyl acetate in Pet Ether as the solvent. In the second solvent system i.e. 25% ethyl acetate in Pet Ether, each fraction loaded showed two spots each. Due to very less quantity of the active fractions obtained after separation, the larvicidal activity of each spot could not be checked. But it was enough in quantity to carry out NMR and IR study.

5.8 NMR (Nuclear Magnetic Resonance) and IR (Infra Red) of Active Fraction:

The spectral pattern of the initial NMR of unprocessed Pet ether fraction and the NMR of organic layer obtained after processing of Pet ether fraction corresponded. IR spectra of the aqueous layer clearly showed the absence of a prominent –OH group pointing that the active compound is not an acid.

The amount of compound obtained after processing was not enough to carry further work. Elucidation of the structure of the active compound needs to be carried out and structure-activity relationships studied. Not much work has been done on study of structure of metabolites with mosquitolarvicidal activity.

5.9 Production and estimation of enzymes of active fungal isolates:

Extracellular enzymes degrading cuticle are associated with virulence of entomopathogenic fungi (Jackson *et al.*, 1985). Conversely no correlation between enzyme activity and pathogenicity was observed in other studies (Rosato *et al.*, 1981). However cuticle degrading enzymes have played a major role in the invasive process of entomopathogenic fungi. Production of a range of extracellular cuticle-degrading enzymes in *M. anisopliae* when grown on locust cuticle in liquid medium was observed by St Leger *et al.* (1986a). The enzymes were produced sequentially corresponding to cuticular components of insects i.e. protein, chitin and lipids with esterases and proteolytic enzymes appearing first followed by N-acetylglucoaminidase (NAGase) while chitinase and lipase were produced last i.e. 3-5 days later. The activity of endoprotease Pr1 appeared to be pathogenicity-determinant as it was produced in large amount during infection and had high cuticle degrading ability (St Leger *et al.*, 1987a, b). Pr1 inhibitors reduced mortality, reduced browning,

invasion of haemolymph and curtailed insect growth rate showing that Pr1 is important for penetration. *Beauveria bassiana*, *Nomuraea rileyi*, *Verticillium lecanii* and *Aschersonia aleyrodis* culture filtrates have shown Pr 1-like enzymes (St. Leger *et al.*, 1987b; El-Sayed *et al.*, 1993; Chrzanowska *et al.*, 2001).

The processing of microbial proteases is simpler compared to animal and plant proteases as they are generally extracellular in nature (Gupta *et al.*, 2002). In the present work protease activity assay carried out in the mosquito-pathogenic *P. citrinum* showed highest protease activity (7.78 U ml^{-1} min^{-1}) at 168 h of incubation and it was comparatively lower and slower than *P. chrysogenum* which showed highest protease activity (12 U ml^{-1} min^{-1}) after 72 h of incubation in a study by Haq *et al.*, 2006. *G. roseum* isolate GUFCC 5039 showed a still lower activity (4.82 U ml^{-1} min^{-1}) at 168 h of incubation. The reduced activity after this period of incubation may be associated with depletion of available nutrients (Romero *et al.*, 1998). Protease activity was detected on second day of incubation in *P. citrinum* in the present study. Similar observations were made in case of *Aspergillus terreus* by Wu *et al.* 2006. The onset of protease production on the second day was correlated by these workers to the increase in pH of the medium above 6.5.

Chitinase is an inducible enzyme (Smith & Grula, 1983) and chitin (St. Leger *et al.*, 1986c) is masked by the proteins, hence only after degradation of cuticular proteins the chitin becomes available which is the reason why chitinase appears late. Inhibitors in the cuticle were thought to be responsible for the absence of chitinase activity during penetration (St Leger *et al.*, 1986b). In our study on *P. citrinum*, chitinase assay carried out showed highest activity (0.012 U ml^{-1} min^{-1}) at 72 h of incubation. In *Trichoderma harzianum* isolate Binod *et al.* (2007) have recorded chitinase activity (0.75 U ml^{-1} min^{-1}) at 24 h of incubation with highest activity (10.2 U ml^{-1} min^{-1}) at 96

h of incubation. Comparatively the chitinase activity in the current study was very low.

It was observed in ultra structural study of the wireworm cuticle, the wax layer on the cuticle hydrolyses just beneath the appressoria and germ tubes of *M. anisopliae* which is attributed to lipase or esterase activity (Zacharuk, 1970; Charnley & St Leger, 1991). Hegedus & Khachatourians (1988) suggested that *in vivo* lipase maybe important in the colonization of the haemolymph rather than the cuticle.

Protease activity was detected in *P. citrinum* at 48 h of fermentation whereas chitinase activity was detected at 24 h of fermentation. Studies on lipase activity also need to be carried out in *P. citrinum*.

5.10 Evaluation of bio-safety of *Penicillium citrinum* to non-target organisms:

Fungi have a very narrow to wide host range, which differs from species to species. *Pandora neoaphidis* which is strictly pathogenic to aphids, does not pose threat to other non-target organisms unlike *B. bassiana*, that infects over 700 arthropod species (Li, 1988) and poses a much bigger risk. A fungus with narrow range is usually an obligate parasite and is seen to cause epizootics, where as a fungus with broad spectrum of hosts is facultative in nature. Testing of infectivity to invertebrate Non-Target Organisms (NTOs) is an important criterion for registration of entomopathogenic fungi as biological control agents (Hall *et al.*, 1982). Bio-safety assessments are predominantly lab based. Entomopathogenic fungi have an edge over the chemical pesticides in the risk posed by them in the environment. Integrated use of fungal control agents is advocated with other control strategies to ensure minimal risk to the NTO (Falcon, 1973; Fuxa, 1987; Goettel & Johnson, 1992). Goettel (1995) opines that it is doubtful whether the artificial augmentation of an indigenous fungus

with wide host range would cause permanent ecological damage as these fungi are ubiquitous, are not very virulent and seldom cause epizootics.

In the present lab based study, biocontrol was achieved by using entomopathogenic fungi targeting an aquatic stage of mosquito life cycle so it was necessary to evaluate the effect of the promising fungal isolate on a NTO that was aquatic in nature. Natural predator of the larvae *Aplocheilus blocki* a larvivorous fish was assessed as a vertebrate model to study the bio-safety of *P. citrinum* in the aquatic environment. Standard 7-day chronic exposure to *P. citrinum* at a high dose (34.76×10^7 spores/ml) did not cause any effect on the external morphology and digestive system especially liver and gall bladder were apparently normal. The gill chamber had green bolus-like material enveloped in mucous but the gills appeared normal. A low mortality of 9% was observed in the experimental aquaria.

The invertebrate NTO, a semi-aquatic insect *L. fossarum fossarum* subjected to standard 4-day chronic exposure of *P. citrinum* conidial suspension at a dose of 3×10^8 spores/ml and observed for ten days did not show any external growth of fungus or sluggishness in the host but a low grade mortality of 4% (1/25) was observed. However whether it was due to fungus was not clear. The bio-safety of crude metabolites of *P. citrinum* (40 µl/ml) tested by acute 20 sec exposure of *L. fossarum fossarum* to the metabolites did not cause sluggishness or mortality for 48 h post exposure though the dose was ten-fold higher than that used against mosquito larvae. The above findings indicate that *P. citrinum* and its metabolites are relatively safe to NTOs but further studies on a wider range of NTO's are needed to arrive at a conclusion.

The prime choice for the control of mosquito vectors the world over is synthetic insecticides but due to the impact of chemical insecticides on environment and the

ever developing resistance in the insects, the use of biological control agents has gained attention. In vector control programmes in India, larvivorous fishes and the bacilli *Bacillus thuringiensis* var. *israelensis* (*Bti*) have been used successfully in large scale. Fungal biocontrol agents are an attractive option and as shown in Table 3 there is an increasing interest in their commercial production for insect control. Entomopathogenic fungi are now being explored to increase arsenal in the fight against vector borne diseases.

In conclusion, this thesis documents the results of an extensive and elaborate work which was accomplished at National Institute of Malaria Research (NIMR), Campal, Panaji for the screening of fungi to test larvicidal activity, effect on NTO and metabolite testing and at Mycolab of Botany Dept. of Goa University Taleigao, Panaji for the isolation, culturing and harvesting of fungi and enzyme studies. At National Institute of Oceanography (NIO), Dona Paula, Panaji work on partial purification and separation of metabolites was carried out. Our studies though are "a drop in the ocean" and of basic nature they provide an insight into the mode of action and bio-efficacy of the indigenously isolated fungi against the vector larvae. This study demonstrates that local strains of *Gliocladium*, *Penicillium*, *Trichoderma* spp. have the potential to kill larvae of vectors like *Anopheles, Culex* and *Aedes*. Lab studies of *P. citrinum* against NTO are encouraging and need to be analyzed further in the field. These mosquito-pathogenic fungi need to be studied further extensively with respect to large-scale production of conidia, the stability of these formulations under laboratory and field conditions needs to be evaluated. Secondary metabolites have shown promising larvicidal activity, the active fraction needs to be studied in detail and has a potential of commercial exploitation.

Chapter 6

SUMMARY

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

~William Lawrence Bragg

6. Summary

Mosquito-borne diseases such as Malaria, Filaria, Yellow Fever, Dengue, Chikunguniya, and Japanese encephalitis cause extensive morbidity and mortality, globally. To curb the vectors of these diseases synthetic insecticides are a popular choice all over the world. However, these have a drawback of causing environmental pollution and also development of resistance in the target insects which together made the search for an alternate tool imperative. Biological control by harnessing natural enemies and entomopathogens is of interest because of their target precision, handler safety, ecological safety and host specificity. Besides, the commercial success of some entomopathogenic fungi in pest control/integrated vector control makes them an attractive option for control of disease vector.

This thesis presents analyses of the pathogenicity and virulence of promising indigenous mosquito-pathogenic fungal strains against larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Aedes aegypti*. The invasive process of mosquito-pathogenic fungi involved, active principles and bio-safety of the promising fungi to non- target organisms were studied. Extensive review of literature was carried out on these different aspects with an attempt to consolidate available data on mosquito-pathogenic fungi.

The entomopathogenic fungi were sourced from 6 different localities of Goa and 10 isolates of fungi were recovered in pure culture either by baiting larvae or sourced from infected mosquitoes and insects. The isolation of fungi from nature and the indigenous strains obtained from Goa University Fungus Culture Collection (GUFCC) indicates that Goa has a rich bio-diversity of entomopathogenic fungi. Using the available taxonomic keys 7 fungal isolates were identified up to species level as follows:

1. Isolate GUFCC 5039: *Gliocladium roseum* Bain.

2. Isolate GUFCC 5040: *Gliocladium roseum* Bain.

3. Isolate GUFCC 5072: *Penicillium citrinum* Thom

4. Isolate GUFCC 5103: *Trichoderma atroviride* P. Karst.

5. Isolate D4: *Aspergillus niger* Tiegh.

6. Isolate D1: *Fusarium oxysporum* Schltdl.

7. Isolate D7: *Fusarium solani* (Mart.) Sacc.

Bioassays of these isolates were carried out using WHO recommended standard method either to screen or test the bio-efficacy of the mosquito-pathogenic fungi. The mortalities were corrected using Abbott's formula wherever necessary. Larval mortality was determined and corrected mortality data were subjected to one way or two-way Analysis of Variance (ANOVA) using software SPSS version 16. The lethal dose/concentration (LD_{50}/LC_{50} and LD_{90}) required to kill 50% or 90% of larvae was calculated by Probit analysis.

Preliminary testing of five fungal isolates for larvicidal activity revealed percent mortalities in the range of 25-100%. *Gliocladium* sp. isolate GUFCC 5044 caused 100% mortality on 24 h exposure, *T. atroviride* caused 70% mortality on 72 h exposure and *P. citrinum* caused 100% mortality on 48 h exposure in *Cx. quinquefasciatus* larvae and hence they were selected for further studies.

Mode of invasion studies in the three fungal isolates revealed different routes of invasion. In *Gliocladium* sp. (isolate GUFCC 5044) cuticle of the *Cx. quinquefasciatus* 3rd instar larva seemed to be the preferred invasion route. The fungus showed profuse mycelial growth on the cuticle and the haemocoel was ramified with fungal mycelia and the organ tissues were disrupted at 24 h exposure. Also, melanization was observed around invading hyphae in the midgut. In *T. atroviride* and *P. citrinum*

invasion seemed to be through the gut of the larva with rapid ingestion of the conidia, packing the gut in 2 h.

In the *An. stephensi* larvae on exposure to *P. citrinum* intense melanization was observed. The degree of melanization differed in exposed individuals. This immune response in *An. stephensi* larvae was faster compared to *Cx. quinquefasciatus* and *Ae. aegypti* larvae.

In the *Cx. quinquefasciatus* larvae exposed to *P. citrinum* extensive mycosis accompanied by emergence of hyphae through the cuticle from the head, thorax, abdomen, anal siphon traversing the gut and haemocoel was observed. Not all the larvae showed mycelial growth; some were dead without any growth (50-60%) indicating role of mycotoxins. No mycelial growth was observed in case of *Ae. aegypti* and *An. stephensi* exposed to *P. citrinum.*

Fate of fungal spores after gut-passage through *Cx. quinquefasciatus* larvae showed that the engested spores of *T. atroviride* were viable as they grew on Corn Meal Agar (CMA) medium.

SEM studies on the *Cx. quinquefasciatus* larvae exposed to *P. citrinum* revealed that conidial attachment to the surface was abundant on the respiratory siphon and anal lobes, slightly lesser in the thoracic region and in patches on the rest of the body. *P. citrinum* conidia were found sparsely on the surface of exposed *Ae. aegypti* larvae.

SEM of dissected gut of *Cx. quinquefasciatus* showed abundant conidia of *P. citrinum* and, of these, a few were germinated with appressorium formation substantiating that infection and invasion through gut was predominant in this vector species.

SEM of the faecal pellet of *Ae. aegypti* and *Cx. quinquefasciatus* larvae showed intact *P. citrinum* conidia enveloped by peritrophic membrane.

Attenuation of virulence is seen in majority of entomogenous fungi *Gliocladium* sp. (isolate GUFCC 5044) showed change in morphology; *T. atroviride* showed reduction in virulence while *P. citrinum* was the most stable and showed no morphological change or loss of virulence over a period of three years on repeated sub culturing in artificial media. Hence *P. citrinum* was chosen for carrying out studies on bio-efficacy, metabolites and safety to NTO and enzyme study.

The bio-efficacy of isolate *P. citrinum* was assessed by performing bioassays against 3rd instar larvae of the three vector species. The highest dose that each vector species was exposed to was 10 x 10^6 spores/ml in *Cx. quinquefasciatus* larvae, in *An. stephensi* larvae 20.02 x 10^6 spores/ml and in *Ae. aegypti* larvae 89.44 x 10^6 spores/ml. This dose in *Cx. quinquefasciatus* larvae caused average mortalities of 72% and 88% on 24 h and 48 h exposure respectively; in *An. stephensi* larvae 54.66% and 84% respectively and in *Ae. aegypti* larvae 48%, 61.33% and 62.7% average percent mortality on 24 h, 48 h and 72 h exposure respectively; showing thereby that *Cx. quinquefasciatus* larvae were most susceptible followed by *An. stephensi* and the least susceptible were *Ae. aegypti*, the latter requiring a much higher dose.

The metabolites of *T. atroviride* tested against *Cx. quinquefasciatus* larvae were larvicidal with LC_{50} value of 26.36µl/ml obtained on 24 h exposure. On screening, *P. citrinum* metabolites resulted in 30% mortality in *Ae. aegypti*, 60% in *An. stephensi* and 82.5% in *Cx. quinquefasciatus* larvae on 24 h exposure in dose range of 4-10 µl/ml.

The results of bioassays with metabolites from submerged cultures of *T. atroviride* and *P. citrinum* showed production of larvicidal toxins. It is assumed that similar toxins could be produced by the conidia in the gut of the larvae.

The age of the conidia seemed to play an important role in the virulence of toxic metabolites. Hence, for separation of active fractions of *P. citrinum* metabolite, 14 d culture was used as this was found to be more virulent compared to the one from 20 d culture. *P. citrinum* metabolites extracted in methanol were highly larvicidal producing 100% mortality in *Cx. quinquefasciatus* on 48 h exposure and *An. stephensi* larvae were found slightly less susceptible as indicated by 94.1% mortality.

Being promising, methanol extract of *P. citrinum* on further separation and partial purification was tested for larvicidal activity against larvae of *Cx. quinquefasciatus* which was the most susceptible to both conidia and metabolites. The Pet ether fraction at a dose of 0.73 mg ml^{-1} showed high larvicidal activity against *Cx. quinquefasciatus* with 98% mortality on 48 h of exposure. The chloroform fraction caused 43% mortality on 48 h exposure at a dose of 0.87 mg ml^{-1}.

Purification of the active fractions methanol, pet ether and chloroform carried by TLC in the present work revealed multiple spots using 30% ethyl acetate in Pet Ether as the solvent. The second solvent system i.e. 25% ethyl acetate in Pet Ether each fraction loaded showed two spots each.

As the mortality in the chloroform fraction was below 50%, it was not investigated further. However, the remaining surviving larvae exposed to this fraction did not pupate indicating growth inhibitory activity and this area warrants further investigation.

The spectral pattern of the initial NMR of unprocessed Pet ether fraction and the NMR of organic layer obtained after processing of Pet ether fraction positively corresponded. IR spectra of the aqueous layer clearly showed the absence of a prominent –OH group pointing that the active compound is not an acid. Further

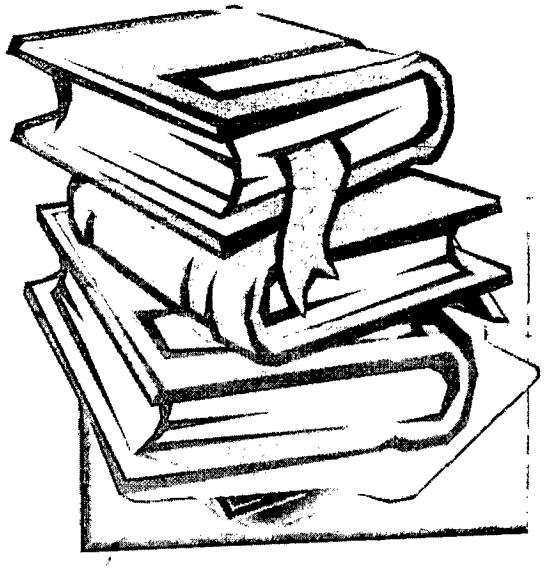
elucidation of the structure of the active compound needs to be carried out and structure-activity relationships studied.

P. citrinum showed highest protease activity (7.78 U ml^{-1} min^{-1}) at 168 h of incubation and *G. roseum* showed lower activity (4.82 U ml^{-1} min^{-1}) at 168 h of incubation. *P. citrinum* when assayed for chitinase, the enzyme production gradually increased with the passage of time and highest enzyme activity (0.012 U ml^{-1} min^{-1}) was obtained after 72 h of incubation and thereafter decreased at 96 h.

Evaluation of bio-safety of *P. citrinum* to non target organisms (NTO) was done by field collection and acclimatization of larvivorous fish *Aplocheilus blocki* in the laboratory and then exposing them to the conidial suspension of *P. citrinum*. Similarly, field collection, establishment and acclimatization in the laboratory of non-target Heteropteran water bugs *L. fossarum fossarum* was done followed by their exposure to conidia and metabolites of *P. citrinum*. In both the NTOs, there was no apparent effect of the *P. citrinum* conidial suspension though low grade mortality (about 4%) was seen in the water bugs. Further, the exposure of *L. fossarum* to metabolites of *P. citrinum* extracted with methanol did not result in any sluggishness or mortality.

As has been mentioned earlier, there was no decline in the pathogenicity of *P. citrinum* on prolonged subculturing on artificial media. The conidial suspension stored in the refrigerator was active even after one year. Also, the virulence of metabolites of *P. citrinum* did not diminish after storage in the refrigerator for a period of thirteen months. The results of larvicidal bioassays showed that all the three test vector species larvae used in this study were susceptible to *P. citrinum* conidia as well as metabolites with susceptibilities in increasing order from *Ae. aegypti*, *An. stephensi* to *Cx. quinquefasciatus*.

Enzyme assays have revealed protease and chitinase production. Effect on NTOs was minimal. Hence *P. citrinum* can be considered as an indigenous good vector control candidate and as such appears to have good scope for development into a mosquito larvicide. It could be formulated and commercialized after extensive and multicentric field trials and confirmation of environmental safety and against NTOs.



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PUBLICATIONS

Publications (to date) arising from the present work:

1. Prabhu D.K. & Kumar A. 2008. *Gliocladium roseum* as a microbial control agent of malaria vector *Anopheles stephensi* Liston and filaria vector *Culex quinquefasciatus* Say. *Kavaka* **36,** 53-56.
2. Prabhu, D.K., Kumar, A. & Bhat, D.J. 2009. Biocontrol efficiency of *Trichoderma atroviride* against larvae of *Culex quinquefasciatus* Say and *Anopheles stephensi* Liston. *Kavaka* **37 & 38,** 7-11.

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Presented in Seminars/Symposium and abstracted:

1. Prabhu, D. K., Bhat, D. J. & Kumar, A. Preliminary Observations on Bioefficacy of *Trichoderma* sp. (strain C37) and *Gliocladium* sp. (strain E20) against filaria vector *Culex quinquefasciatus* Say, in Goa, India. **In:** National Seminar on "*Microbial Biodiversity-A Source of Innovation in Biotechnology*" during 27th - 29th May 2004 at Tropical Botanic Garden and Research Institute, Thiruvananathpuram, Kerala, India.
2. Prabhu, D.K., Kumar A. & Bhat, D.J. Efficacy of mosquito larvicidal fungus *Pencillium citrinum* Thom and its bio-safety to non-target semi-aquatic Heteropteran insect *Limnogonus (Limnogonus) fossarum fossarum.* **In:** "*X International Symposium on Vectors and Vector Borne Diseases*" during 4th - 6th November 2009 at Goa, India.

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Gliocladium roseum as a microbial control agent of malaria vector Anopheles stephensi Liston and filaria vector Culex quinquefasciatus Say.

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ABSTRACT

Gliocladium roseum was isolated from an aphid collected from *Cassia tora* at Pernem, Goa. The bio-efficacy of the fungal isolate grown for 14 days against 3rd instar larvae of mosquito vectors *Anopheles stephensi* and *Culex quinquefasciatus* using a conidial dose of 10.52×10^6 spores/ml resulted in 92.8% and 71.9% mortality respectively in 72 h. The difference in mortality in *An. stephensi* larvae at 24, 48 and 72 h was not significant between the 5 replicates ($F = 1.091$, $p = 0.394$), while it was significant in *Cx. quinquefasciatus* ($F = 9.333$, $p = 0.014$) with increase in the time of exposure. Results suggest that *G. roseum* GUFCC 5040 has potential for use in mosquito control programmes.

Key words: Biocontrol of mosquito; malaria control

INTRODUCTION

In India there are estimated 29 million cases of filariasis and about 1.5 million reported cases of malaria annually (http://w w w.searo.who.int/EN/Section313). *Anopheles stephensi*, a sub-tropical species, distributed throughout the Middle East and South Asia region is one of the primary vectors of malaria (Kumar *et al.*, 2007). *Culex quinquefasciatus,* breeding in organically rich polluted waters, is a primary vector of *Wuchereria bancrofti*, the causative organism of Bancroftian filariasis (Curtis *et al.*, 1981). Vector control involving major drainage works is a costly affair and rapid development of resistance to insecticides has made search for bio-control agents imperative. Larvivorous fishes and bacteria have been deployed in mosquito control programmes but have some limitations (Federici, 1995; Kumar and Hwang, 2006). Emergence of low level of resistance in the vectors to bacterial control agents and the commercial success of some entomopathogenic fungi in pest control also makes them an attractive vector control proposition (Silva-Filha, *et al.*, 1995; Butt *et al.*, 2001).

Amongst the entomopathogenic fungi, Hyphomycetes have been reviewed extensively as bio-control agents (Ferron, 1978; Tanada and Kaya, 1993; Hajek and St. Leger, 1994; Wraight and Carruthers, 1999; Keshava Prasad and Bhat, 2007). A recent report focuses on the use of entomopathogenic fungi for mosquito control (Scholte, *et al.*, 2004). *Gliocladium roseum* Bainier is a soil fungus with broad spectrum activity. *G. roseum* is a mycoparasite of plant pathogenic fungi like grey mold *Botrytis cinerea*, Cucumber black root rot fungus, *Phomopsis sclerotioides* and *Verticillium* sp. (Barnett and Lilly, 1962; Moody and Gindrat, 1977; Keinath *et al.*, 1991). *G. roseum* is also known to parasitize nematodes (Zhang, *et al.*, 2008). Recently, it was shown to produce myco-diesel hydrocarbons (Strobel, *et al.*, 2008). In the present study, the potential of *G. roseum* as a mosquito control agent has been investigated.

MATERIALS AND METHODS

Isolation and Identification of the Fungal isolate

Gliocladium roseum was isolated from an aphid collected from *Cassia tora* at Pernem, Goa by direct isolation method and maintained in Goa University Fungal Culture Collection (isolate No.GUFCC 5040). The pure culture of the fungus was maintained on Malt Extract Agar (MEA) medium (Himedia Laboratories Pvt. Ltd.: 2.5 g dehydrated malt extract and 20 g agar in 1000 ml distilled water). The isolate was subcultured on Corn Meal Agar (CMA) medium (Himedia Laboratories Pvt. Ltd.: 17g cornmeal agar in 1000 ml distilled water).

Test Organisms

An. stephensi and *Cx. quinquefasciatus* 3rd instar larvae cultured and maintained at National Institute of Malaria Research, Field Station, Goa, were used in the studies. They were maintained at a temperature of 25 ± 2°C, relative humidity of 70 ± 5% and a photoperiod:scotoperiod of 14:10 h (light:dark).

Preparation of Conidial Suspension

For obtaining conidia in large quantity, *G. roseum* was grown on CMA. The culture Petri plates were flooded with sterile 0.05% Tween 80 and spores harvested at the end of 14 days. The suspension centrifuged at 6000 revolutions per min (rpm) for 10 min yielded a pellet which was washed in sterile distilled water, re-suspended in minimum amount of water and vortexed. The spore concentration was determined using improved Neubauer's haemocytometer (Goettel and Inglis, 1997).

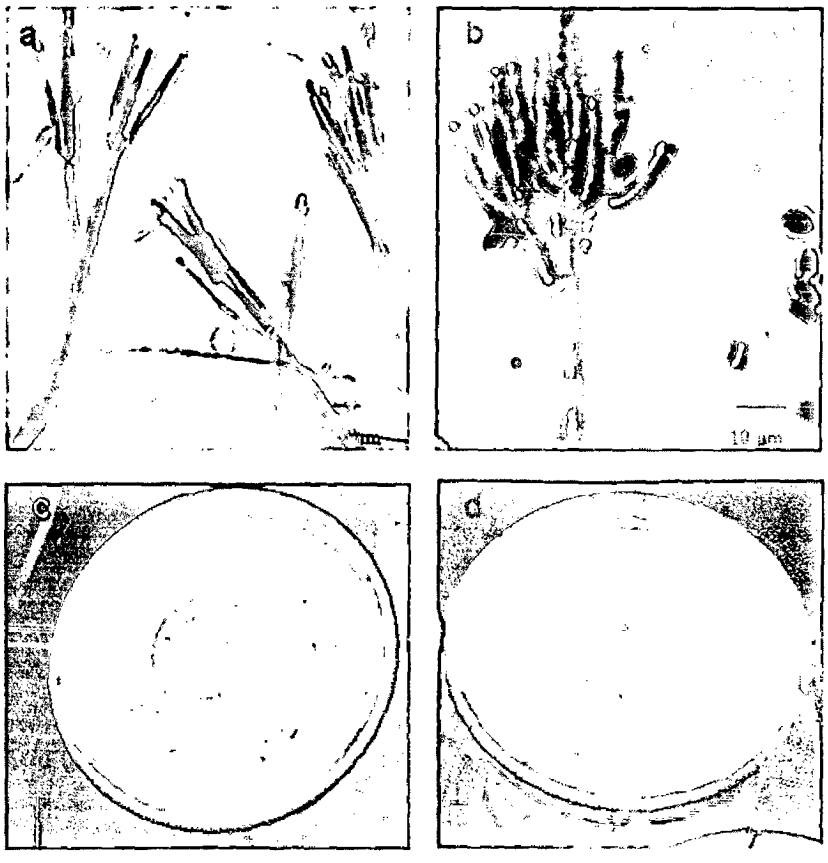

Fig. 1 a, b. *G. roseum* GUFCC 5040 a. Primary conidiophores of *Verticillium* - type 40x b. Secondary conidiophores of Penicillate type 100x (scale bar = 10 µm) c, d. Bioassay of *G. roseum* against *Cx. quinquefasciatus* 3rd instar larvae c. Control d. Experimental

Bioassays of *G. roseum* against mosquito larvae

The bio-efficacy of *G. roseum* against *An. stephensi* and *Cx. quinquefasciatus* 3rd instar larvae was assessed using a WHO (1996) recommended standard method. Bioassays were set up in 500 ml plastic bowls with a wide surface area in a final volume of 50 ml sterile distilled water with the dose of 10.52×10^6 spores/ml. In all, 5 replicates each with 20 healthy 3rd instar larvae were set up. Three control replicates without the spore suspension were concurrently maintained for 72 hours. The bowls were covered with nylon net secured with a rubber band from outside margin (Fig 1.c, d). Larvae were fed with 20-25 mg baby food Farex ™ daily. Mortality counts were recorded at 24 h interval based on the number of live larvae remaining in each bowl and assays were terminated at 72 h. Moribund and dead larvae were examined individually under a binocular microscope. Corrected mortality was calculated using Abbott's formula when control mortality was between 5 and 20% (Abbott, 1925).

The percent mortality observed in each batch of larvae per replicate for 24 h, 48 h and 72 h was subjected to analysis of variance (ANOVA) (Table 1).

Table 1 a: ANOVA table to ascertain the significance of larvicidal activity of conidial suspension (14 d) of *Gliocladium roseum* against *Anopheles stephensi* 3rd instar larvae with respect to different time intervals

Anova Table					
Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Treatments	2	66.667	33.333	1.091	0.394
Error	6	183.333	30.556	–	–
Total	8	–	–	–	–

Coefficient of Variation = 6.142

Differences in mortality at different Treatments found to be Non Significant

Table 1 b: ANOVA table to ascertain the significance of larvicidal activity of conidial suspension (14 d) *Gliocladium roseum* against *Culex quinquefasciatus* 3rd instar larvae with respect to different time intervals

Anova Table					
Source of variation	Degrees of freedom	Sum of squares	Mean sum of squares	F cal	F prob
Treatments	2	1088.889	544.444	9.333	0.014 *
Error	6	350.000	58.333	–	–
Total	8	–	–	–	–

Coefficient of Variation = 12.970

Treatments found Significant at 5% level of Significance

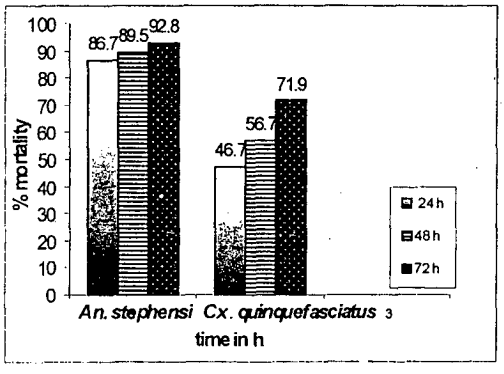

Fig. 2. Susceptibility of 3rd instar mosquito larvae to *Gliocladium roseum* isolate GUFCC 5040

F cal = F calculated value; F prob = F probability; * = significant

RESULTS AND DISCUSSION

Colonies of the fungus on CMA were circular, pale creamy white with smooth hairy margin, attaining a diam. of 3-4 cm in 7 days, slimy, concentric rings of wet globules of conidia after initial floccose growth with thick mycelial strands were bearing short conidiophores and wet heads of conidia. Reverse of the colony was colourless. The mycelium was composed of smooth, hyaline, septate, branched, 2-3 µm wide hyphae. Conidiophores were of two types: Primary conidiophores of *Verticillium*- type (Fig. 1.a), and secondary conidiophores of penicillate type (Fig. 1.b), mononematous, smooth, sometimes pitted, septate, branched, thin walled, hyaline, mostly 100-150 %m in length, 60-200 µm long, 3-4 µm at the middle and 2-3 µm at the base of penicillia, the base of the conidiophore narrowed. The conidiogenous cells were phialidic, discrete, $10-25 \times 2-3$ µm tapering towards the tip. Conidia were slimy, solitary, navicular hyaline, $4-7 \times 3-4$ µm. Following Domsch *et al.* (1980) the isolate was identified as *Gliocladium roseum* Bain. 1907.

In the present study *G. roseum* caused 86.7%, 89.5% and 92.8% mortality in *An. stephensi* 3rd instar larvae on 24 h, 48 h and 72 h exposure respectively to conidial suspension prepared from 14 day old culture. *Cx. quinquefasciatus* 3rd instar larvae were comparatively less susceptible with mortality of 46.7%, 56.7% and 71.9% when exposed for 24 h, 48 h and 72 h. However, an increase in exposure time caused 25.2% increase in mortality in *Culex* larvae compared with 6.1% increase in mortality in *An. stephensi* larvae (Fig. 2).

In appearance, the dead *Culex* larvae turned pale and shriveled whereas, *Anopheles* larvae gradually turned black, which is an indication of melanization, which is a process of cellular defense response of the host against a pathogenic fungus as has been earlier reported (Ferron, 1978). It has been previously shown that when pathogenic fungus succeeds in penetrating cuticle, the plasmatocytes which are present in the hemolymph accumulate around the fungus and give rise to melanization. In a study on the isolates of *Beauveria* spp. a fast and intense melanization of the cuticle of the integument and of tracheal wall in the larvae of the lepidopteran *Galleria mellonella* was observed. Further, role of melanizing macromolecules which arc vivotoxins secreted during the mycosis, has also been observed in the *Galleria* larvae using an isolate of *Beauveria bassiana* (Fuguet and Vey, 2004).

On exposure to 14 d conidial suspension of *G. roseum* with increase in exposure time in *An. stephensi* 3rd instar larvae mortality difference was not significant between the 5 replicates ($F = 1.091$, $p = 0.394$), while it was significant in *Cx. quinquefasciatus* 3rd instar larvae ($F = 9.333$, $p = 0.014$). This indicates that Anopheline and Culicine host responses to *G. roseum* infection in larvae were variable although both were susceptible to the fungus. *An. stephensi* larvae are more susceptible to *G. roseum* compared to *Cx. quinquefasciatus* 3rd instar larvae. Results suggest that *G. roseum* GUFCC 5040 has potential for use in mosquito control programmes.

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Biocontrol efficiency of *Trichoderma atroviride* against larvae of *Culex quinquefasciatus* Say and *Anopheles stephensi* Liston

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ABSTRACT

Conidial suspension of *Trichoderma atroviride* isolated from larvae of *Anopheles* sp., at a concentrations of 6.9×10^6 spores/ml 11.6×10^6 spores/ml, killed 65-70% *Culex quinquefasciatus* and 25% *Anopheles stephensi* 3rd instar larvae on 72 h exposure. Thin sections of infected larvae respectively of *Cx. quinquefasciatus* showed accumulation of conidia in the gut. Tests conducted revealed that the conidia were viable even after passage through the gut of *Cx. quinquefasciatus* and in the faecal pellets of *An. stephensi*. The LC_{50} value of metabolites from cell free extract of *T. atroviride* against *Cx. quinquefasciatus* larvae was 26.36 µl /ml at 24 h. Mortality difference was significant within the 5 replicates/dose ($F = 6.227$, $p < 0.002$), while it was highly significant between the 3 doses ($F = 244.342$, $p < 0.001$). It is, therefore, projected that *T. atroviride* could be a potential biolarvicidal material.

Keywords: *Trichoderma atroviride*; entomopathogenic fungi; *Culex quinquefasciatus*; *Anopheles stephensi*; *Trichoderma* metabolites

INTRODUCTION

Bancroftian filariasis and malaria are world's most predominant tropical infectious diseases. Of the 128 million cases of lymphatic filariasis estimated globally, 91% are caused by *Wuchereria bancrofti* (Michael and Bundy, 1997). *Cx. quinquefasciatus* is the principal vector of bancroftian filariasis in India breeding in polluted waters. *An. stephensi*, the malaria vector in urban areas, is responsible for serious malaria outbreaks in Goa (Dash *et al.*, 2008). In the adult *Culex* mosquitoes rapid build up of resistance to synthetic pyrethroids and emergence of insecticide resistance in species of *Anopheles* has led to the search for alternate vector control strategies.

Entomopathogenic fungi are considered as potential alternatives for chemical insecticides. Reports are available on effective use of entomopathogenic hyphomycetes and their development as microbial control agents (Ferron, 1978; Tanada and Kaya, 1993; Hajek and St. Leger, 1994; Scholte *et al.*, 2004; Keshava Prasad and Bhat, 2007).

Many entomopathogenic fungi produce insecticidal toxins in liquid culture (Charnley, 2003). Tolypin from *Tolypocladium niveum* was reported to be larvicidal to the larvae of mosquitoes and blackflies (Matha *et al.*, 1988). The depsipeptide beauvericin from some *Fusarium* spp. was toxic to Colorado potato beetle (Gupta *et al.*, 1991) and from *Beauveria bassiana* caused ultrastructural changes especially in the midgut epithelium and mortality in the *Culex pipiens autogenicus* larvae (Zizka and Weiser, 1993). Extracellular metabolites of fungi active against mosquito larvae have been screened in India (Vijayan and Balaraman, 1991; Mohanty and Prakash, 2004).

Trichoderma atroviride, a filamentous soil fungus, is known to function as biocontrol agent for a wide range of economically important aerial and soil borne plant pathogens (Papavizas, 1985; Chet, 1987). The present study describes activity of *T. atroviride* and its metabolites against the mosquito vectors.

MATERIALS AND METHODS

Test Organism

Cx. quinquefasciatus larvae cultured and maintained by the National Institute of Malaria Research, Field Station, Goa, at a temperature 25 ± 2°C, relative humidity 70 ± 5% and photoperiod : scotoperiod 14:10 h (light : dark) were used in the present study.

Mycopathogen

An isolate of *Trichoderma* was recovered from 3rd instar larva of *Anopheles* sp. inhabiting curing water at a construction site in Cuncolim, Goa, by direct isolation method and maintained at Goa University Fungal Culture Collection (GUFCC No. 5103). The isolate was maintained on Malt Extract Agar (MEA) medium (Himedia Laboratories Pvt. Ltd.: 2.5 g dehydrated malt extract and 20 g agar in 1000 ml distilled water). For all experiments, the isolate was

subcultured on Corn Meal Agar (CMA) medium (Himedia Laboratories Pvt. Ltd.: 17g cornmeal agar in 1000 ml distilled water). Following Gams *et al.* (1987), the isolate was identified as *Trichoderma atroviride* P. Karsten.

Cultural Characters of *T. atroviride*:

Colonies of the fungus on CMA (Fig.1a) were circular, with rhizoidal margin, attaining a diam of 8-9 cm in 7 days, slimy conidia in patches, initially white, rapidly turning to green, with watery exudates on the surface, conidiation appeared granular with age, initially glaucous, reverse uncoloured. The mycelium was composed of smooth, hyaline, septate, freely branched thin-walled hyphae, 4-7 µm wide. Unicellular, subglobose chlamydospores were present within the hyphal cells. Conidiophores were mononematous, relatively narrow and flexous with usually paired short primary branches arising at regular interval, which extensively rebranched, coarse with tubercles, septate, thin walled hyaline, $30-40\times2-3$ µm (Figure 1b). The conidiophores terminated with few phialides and with their branches at right angles assumed a pyramidal shape. The conidiogenous cells were phialidic, discrete, rough, hyaline, $5-7\times3-3.5$ µm, lageniform, with a very narrow beak like distal tip; length/width ratio of phialides was $1.7-2.3$ µm (Fig.1c) Conidia were globose to subglobose, smooth forming sticky balls of 10 – 15 on each conidiogenous cell, green, $2-3$ µm diam (Fig.1d).

Preparation of Conidial Suspension:

For obtaining conidia in large quantity, the isolate was grown on CMA. The culture plates were flooded with sterile 0.05% Tween 80 and spores harvested. The suspension centrifuged at 6000 rpm for 10 min yielded a pellet which was washed in sterile distilled water, re-suspended in minimum amount of water and vortexed. The spore concentration was determined using improved Neubauer haemocytometer (Goettel and Inglis, 1997).

Preparation of Cell Free Extract (CFE):

Two 250 ml conical flasks containing sterilized 100 ml of Glucose Peptone Yeast Medium (glucose 10 g, peptone 10 g, yeast extract 4 g, soluble starch 10 g, distilled water 1000 ml, pH 5.5) were inoculated with conidia of *T. atroviride* from 14 d old culture and incubated at 25.8 ± 2°C for 10 d at 150 rpm on an Orbitek rotary shaker. The buff coloured culture obtained when sonicated using electronic sonicator for 2 min yielded a homogenous brown coloured liquid. The liquid was centrifuged for 20 min at 5500 rpm, the residual pellet discarded and the CFE used for bioassay. An uninoculated culture medium served as control.

Bioassays

Larvicidal activity of *T. atroviride* against *Cx. quinquefasciatus* 3rd instar larvae was assessed using a standard method recommended by the World Health Organization (WHO, 1996). Bioassays were set up in 500 ml plastic bowls with a wide surface area in a final volume of 50 ml sterile distilled water with the desired active dose of 6.9×10^6 spores/ml. In all, 5 replicates each with 20 healthy 3rd instar larvae were set. Three controls, without spore suspension, were maintained. The bowls were covered with nylon net secured by a rubber band. Larvae were fed with 20 – 25 mg baby food Farex™. Assays were terminated at 72 h and mortality counts made based on the number of live larvae remaining in each bowl. Moribund and dead larvae were examined individually under a microscope.

Similar assay with a dose of 11.6×10^6 spores/ml was carried out against *An. stephensi* 3rd instar. Bioassay of conidial suspension with dose of 11.6×10^6 spores/ml from 28 d culture, was carried out against *Cx. quinquefasciatus*

Studies on mode of action:

Stereomicroscopic studies showed presence of green matter within the exposed larva like a tube. The larvae were dissected with a sterilized blade to expose the contents of the gut lumen.

Light microscopy of *Culex* larvae:

The location of conidia in the gut of *Cx. quinquefasciatus* after ingestion was studied under a light microscope. After 48 h exposure the treated and control larvae were fixed in Altmann's fixative for 2 h (Gatenby and Beams, 1950) and sectioned in a Leica M-800 cryostat at −14°C to obtain 20 µm thick longitudinal sections. They were air dried, stained with lactophenol mixed with cotton blue and observed under a binocular microscope.

Viability of *T. atroviride* conidia in the gut of *Cx. quinquefasciatus*:

The viability of *T. atroviride* conidia in the gut of *Cx. quinquefasciatus* following their ingestion and excretion by the larvae was studied by glutting with India ink (Goettel, 1988 b).

Three replicates with 10 larvae per replicate were used.

Bioassays with metabolites of *T. atroviride*:

Screening crude metabolites in the CFE of *T. atroviride* against *Cx. quinquefasciatus* larvae resulted in mortality above 60% in 24 h. Based on these results main assays were set with three different concentrations of metabolites 1 µl/ml, 10 µl/ml and 100 µl/ml. Five replicates of 10 *Cx. quinquefasciatus* larvae per replicate per dose were set in 500 ml plastic bowls with total volume made to 50 ml with deionised water. Concurrently three controls with respective concentration of media were maintained. In each bowl 15 mg baby food Farex ™ was provided as food. The bowls were covered with nylon net secured with a rubber band. Larval mortality was recorded at 24 h and 48 h. Experimental tests that demonstrated more than 20% control mortality were discarded and repeated. When control mortality reached between 5-20%, the mortality observed was corrected by Abbott's formula (Abbott, 1925).

STATISTICAL ANALYSIS

Results of efficacy of *T. atroviride* metabolites assessed against *Cx. quinquefasciatus* were analyzed by probit analysis (Finney, 1971). The concentrations caused 14 – 100% mortalities in the larvae and LC_{50} values were calculated with their fiducial limits at 95% confidence level (Table 1). The relation between probit and log concentration was established as probit equations. Percentages of larval mortality were determined and subjected to analysis of variance (ANOVA) (Table 2) using SPSS version 16 software.

RESULTS AND DISCUSSION

The bioassay of *T. atroviride* conidial suspension against 3rd instar larvae of *Cx. quinquefasciatus* resulted in 65% – 70% mortality and in *An. stephensi* 25% were killed. In a previous study (Keshava Prasad *et al.*, 2000) 65% mortality was seen in 2nd instar *Cx. quinquefasciatus* on 120 h exposure to *T. atroviride* (GUFCC 5103). Comparatively the susceptibility of the 3rd instar *Cx. quinquefasciatus* seems to be more in the present as mortality was achieved on 72 h exposure. *An. stephensi* appears less susceptible though the dose was almost two-fold higher. Under stereomicroscope the alimentary canal in both *Culex* and *Anopheles* larvae appeared packed with green coloured matter probably green spores of *T. atroviride*. Dead larvae had distorted body with head separated from the thorax. The surviving larvae entered the 4th instar but breadth wise they were thinner than the control larvae. The larvae in the control however were active, feeding voraciously, healthier and developed normally.

Table1. Larvicidal activity of secondary metabolites of *Trichoderma atroviride* against the larvae of *Culex quinquefasciatus*

Time (h)	Probit equation	LC_{50} (µl/ml)	95% Confidence Interval LBUB	x^2
24	$Y = -1.600 + 1.126\,X$	26.36	.408 1.844	1.205
48	$Y = -1.057 + 1.373\,X$	5.886	.551 2.195	1.375

LB = Lower Bound; UB = Upper Bound

Table2: ANOVA table to see the significance of larvicidal activity of metabolites of *Trichoderma atroviride* against *Culex quinquefasciatus larvae*

Source of variation	Degrees of freedom df	Sum of squares ss	Mean sum of squares ss	F cal	F prob
Replications	4	430.048	107.512	6.227	0.002*
Treatments	5	16531.740	3306.348	191.502	0.000**
Factor A	2	8437.281	4218.640	244.342	0.000**
Factor B	1	346.363	346.363	20.061	0.000**
A X B	2	7748.097	3874.048	224.383	0.000**
Error	20	345.307	17.265	–	–
Total	29	–	–	–	–

Coefficient of Variation = 9.185

Factor A = Dose; Factor B =Time; df = degrees of freedom; SS = Sum of squares; Mean SS = Mean sum of squares; F cal = F calculated value; F prob = F probability ;* = significant, ** = highly significant.

Al-Aidroos and Roberts (1978) observed gut invasion of larvae of *A. aegypti* by *M. anisopliae*, confirming that mosquitoes are vulnerable to this mode of attack. Reports of insect mortality due to the toxic activities of large number of ingested but ungerminated conidia in the mosquito larvae are available (Roberts, 1970; Crisan 1971). In the present study the dissected gut showed the conidia in the chyme, light microscopy revealed the presence of the conidia throughout the alimentary canal (Figure 1.d) in the longitudinal sections (L.S) of *Cx. quinquefasciatus* larvae, it was also noted that the tissue had taken up blue colour but the conidia were unstained (Figure 1.e) right from foregut uptil the hindgut, probably they were coated with some secretions, many had assumed crescent shape, slight flattening of the conidia observed maybe due to mechanical pressure, a tendency of the conidia to remain more towards the gut epithelium was seen (Figure 1.f). Though the conidia had remained for 48 h in the gut germination or hyphal formation was not seen. The viability experiments showed that after being held for more than two hours in the gut, the conidia were viable. They were viable prior to ingestion. The conidia retained their viability ensuing ingestion and excretion. After holding in distilled water for 24 h when plated on CMA the conidia were viable.

Similarly in *T. cylindrosporum* (Goettel 1988a, b) the viability of spores was not reduced after a 2.5 h passage through the larval gut of the 2nd instar *A. aegypti* and at the time of death in the larvae exposed to higher concentrations of conidia, less extensive mycoses was observed. In concurrence with Soares (1982) in the present study mortality in larvae maybe attributed to nutritional stress or it maybe due to production of toxic metabolites. Bioassay with a higher dose of conidial suspension from 28 d culture against *Cx. quinquefasciatus* did not cause any mortality, showing that higher concentrations may not be causing death. Green faecal pellets were observed showing that the spores were ingested and egested. The results of bioassays with metabolites from CFE of 10 d submerged culture indicate production of larvicidal toxins. Similar toxins might be produced by the conidia in the gut of the larvae and the age of the conidia seems to play an important role. In *Anopheles* larvae the conidia from the faccal pellets plated on antibiotic loaded CMA germinated.

Bioassays with metabolites from the CFE of *T. atroviride* against *Cx. quinquefasciatus* larvae with doses of 1 µl/ml, 10 µl/ml and 100 µl/ml caused percent mortalities of 14%, 22% and 76% respectively after 24 h exposure and 24%, 46% and 99%, respectively after 48 h exposure. Control mortality ranged from 0 – 6.66%. LC_{50} for 24 h = 26.36 µl/ml and LC_{50} for 48 h = 5.886 µl/ml. Mortality difference was significant within the 5 replicates/dose ($F_6 = 6.227$, $p = 0.002$), while it was highly significant between the 3 doses ($F = 244.342$, $p < 0.001$). The significance in mortality was high with increase in exposure time from 24 h to 48 h ($F = 20.061$, $p = 0.000$). In their studies Vijayan and Balaraman (1991) obtained the LC_{50} values of fungal metabolites from 17 species in the range of 3 – 24 µl/ml for *Cx. quinquefasciatus* 3rd instar larvae on 48 h exposure whereas in the present LC_{50} value was achieved on 24 h exposure with slightly higher dose of 26.36 µl/ml. The LC_{50} value of *Chrysosporium tropicum* (Priyanka and Prakash, 2003) evaluated against 3rd instar *Cx. quinquefasciatus* larvae was 79 µl/ml which is three-fold higher than *T. atroviride*. A crude extract of tolypin caused 100% mortality in the larvae of *Cx. pipiens* and *An. maculipennis* at a concentration of 100 µl /ml (Weiser and Matha, 1988).

Our results show that *T. atroviride* (GUFCC 5103) and its metabolites are highly efficacious against the *Cx. quinquefasciatus* larvae and has potential which needs to be exploited against other vector larvae.

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