HOST-PATHOGEN INTERACTION IN *METARHIZIUM ANISOPLIAE* AND ITS INSECT HOST *HELICOVERPA ARMIGERA*

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BY

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UNDER THE GUIDANCE OF

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Dedicated to my beloved parents

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CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Host-pathogen interaction in *Metarhizium anisopliae* and its insect host *Helicoverpa armigera*"** submitted by Ms. Manisha Kapoor was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled, "**Host pathogen interaction in *Metarhizium anisopliae* and its insect host *Helicoverpa armigera***" submitted by me for the degree of Doctor of Philosophy to the University of Pune, is the record of work carried by me at Biochemical Sciences Division, National Chemical Laboratory, Pune - 411008, Maharashtra, India, under the supervision of Dr. M. V. Deshpande (research guide) and Dr. A. Sen (research co-guide). The work is original and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

Manisha Kapoor
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Manisha Kapoor

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AFM	Atomic Force microscopy
d	Day
GlcNAc	N-Acetylglucosamine
g, mg µg	gram, miligram, microgram
h,min, sec	Hour(s), Minute(s), Second(s)
kD	kilo Dalton
L, ml, µl	Liter, Mililiter, Microliter
M, mM, µmol	Molar, Milimole, Micromole
MALDI-TOF-MS	Matrix assisted laser desorption ionization-Time of flight-Mass Spectrometry
OD	Optical density
PAGE	Polyacryl amide gel electrophoresis
RH	Relative humidity
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
w/v	Weight by volume

ABSTRACT

The introduction of organochlorine insecticides during the 1940's offered a revolution in the efficiency of crop protection and vector control practices. Although the scientific community was drawing attention to governments regarding the ill effects of synthetic pesticides, public concern was galvanized after dramatic revelations of the harmful effects of synthetic pesticides published in Rachel Carson's "Silent Spring" (Carson, 1962). Nevertheless, the use of pesticides has increased manifolds between 1950 and 2010 with crop pests still costing billions of rupees annually in terms of yield loss to agricultural production. The widespread and injudicious use of synthetic pesticides has been and still is a threat to the environment and human health. Indiscriminate use of pesticides leads to residues in the soil and the depletion of natural enemies besides serving as a powerful selection pressure for altering the genetic makeup of a pest population leading to the development of resistance. In India, over 700 species of insect pests have developed resistance not only to individual chemicals but also to groups of chemicals, a notable example being the gram pod borer, *Helicoverpa armigera* which has attained the status of a 'national pest'. Despite the enormous benefits of the green revolution, which required high inputs like fertilizers, pesticides and irrigation, pests continue to be a major biotic constraint. A general estimate of annual crop losses due to insect pests amounts to Rs. 60,000 crores per year. Globally, *H. armigera,* a polyphagous pest causes a yield loss worth about US $ 2 billion annually (ICRISAT, 2003). In India, the loss due to this pest on pigeon pea and chickpea was estimated at US $ 200 million annually.

In recent years, the need to increase food production to meet the demands of a rapidly growing population from limited land resources and given the scenario of detrimental effects of synthetic pesticides to control insect pests and vectors, it becomes imperative to search for alternative methods of control which are ecologically sound, reliable, economical and sustainable. Biological control offers a suitable alternative and includes the use of parasites, predators and microbial pathogens. Naturally occurring entomopathogens are important regulatory factors of insect populations. The potential of pathogens in applied biological control exceeds that of many other methods, including the use of parasites and predators. Bacteria, fungi, viruses, protozoans and nematodes have been successfully used in the control of several insect pests.

Compared to other microorganisms, fungi are known to infect a broad range of insects including lepidopterans, hymenopterans, coleopterans and dipterans often resulting in natural epizootics. An added advantage in using entomopathogenic fungi is that infectivity is by contact and the action is through penetration. These fungi comprise a heterogenous group of over 100 genera with approximately 750 species, reported from different insects. The most important fungal pathogens are *Metarhizium* spp., *Beauveria* spp., *Nomuraea rileyi*, *Verticillium lecanii* and *Hirsutella* spp. *Metarhizium anisopliae* (Metchnikoff) Sorokin is the second most widely exploited entomopathogenic fungus in biocontrol programs. It is known to attack over 200 species of insects. Present study is an attempt to understand the interaction between the widely used biocontrol agent i.e. *M. anisopliae* and its highly destructive insect host *H. armigera.* The findings of the study will be useful in the development of effective biocontrol strategy for the management of different pests. The results of the study are summarized as follows:

Chapter 1. In **Introduction and review of literature** insect pest control, existing chemical control strategies and market status of the different chemical control agents are discussed. Taking into account the disadvantages and limitations of synthetic pesticides, alternative strategies to control insect-pests are enumerated. Emphasis is laid on microbial pathogens, in particular entomopathogenic fungi. Factors affecting their successful establishment on a host are discussed *viz.*, the role of insect cuticular hydrocarbons during the initial stages and the subsequent role of cuticle degrading enzymes in the virulence of entomopathogenic fungi. In addition, surface characterization of fungal conidia which aid in identifying the parameters crucial for successful development of a mycoinsecticide is discussed.

Chapter 2. The second chapter on **Materials and Methods** describes the chemicals used, their source, the analytical methods used for measuring the biophysical and biochemical parameters as well as the various bioassays conducted in the study**.** The details of growth and maintenance of entomopathogenic fungus, *M. anisopliae* and solid state fermentation for production of conidia are outlined. Microbial techniques like germtube formation and appressorium formation are described. Assays for estimation of total chitinase activity, chitin deacetylase, chitosanase, lipase, protease are described. Biophysical characteristics of infective propagules (conidia and

blastospores) by contact angle measurements, Zeta-potential and atomic force microscopy are described. The nature of the rodlet layers on conidia were analysed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently characterized by matrix assisted laser desorption ionisation – time of flight spectrometry (MALDI-TOF). Morphological changes involved in the gradual establishment of *M. anisopliae* on insect hosts, *H. armigera* and *Ae. aegypti* were studied with scanning electron (SEM) while the degradation of cuticular hydrocarbons of larvae of *H. armigera* were analysed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). A brief description is given on rearing of *H. armigera* and *Ae. aegypti* along with details on the procedures involved in specific bioassays with the larval and adult stages of the above mentioned insect species.

Chapter 3. Screening of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* - The chapter discusses experiments on evaluation of sixty three cultures of *Metarhizium* isolated from soil (53) and insect hosts (10) against *H. armigera*. Preliminary bioassays revealed mortality of *H. armigera* to vary between 20-97% within 14 d and ten isolates were selected for further evaluation on the basis of percent mortality. Lethal time to kill 50% population (LT_{50}) was determined for these ten *Metarhizium* isolates. In addition, extracellular production of cuticle degrading enzyme (CDE) complex, comprising enzyme activities for chitinase, chitin deacetylase (CDA), chitosanase, protease and lipase, was also estimated. *Metarhizium* isolates M34412 and M81123 showed lowest LT_{50} (3.3 d), whereas LT_{50} values were 3.5, 3.6, 4.1 for isolates M34311, M91629 and M91427, respectively. The remaining isolates had relatively higher LT_{50} values. These five strains with lower LT_{50} values were further checked for LC_{50} which was found to be in the range of 1.4×10^3-5.7×10^3 conidia/mL. Out of the five isolates, three isolates (M34412, M34311 and M81123) were selected on the basis of higher conidia production (60-75 g/kg rice), faster sedimentation time (ST_{50}) (2.3-2.65 h in 0.1% (w/v) Tween 80) and higher percent viability (92-97%). The three isolates were identified as *M. anisopliae* by ITS sequencing. The field performance of the three isolates against *H. armigera* was evaluated in a chickpea crop. The percent efficacies with the three *M. anisopliae* strains were from 65 to 72%, which was comparable to the chemical insecticide,

endosulfan (74%). On the basis of these results, *M. anisopliae* M34412 was investigated in detail for the pathogenesis in *H. armigera*.

Chapter 4. *Metarhizium anisopliae* pathogenesis in *Helicoverpa armigera* and factors involved in their interaction -

A. Pathogenesis of *M. anisopliae* in *H. armigera*- Pathogenesis of an insect by entomopathogenic fungi occurs by a series of events. Scanning electron microscopic (SEM) analysis of infected *H. armigera* showed that preferred attachment sites of conidia of *M. anisopliae* were insect setae, hair socket joints, legs. Conidia adhered and consolidated to the insect integument within 0 to 6 h and initiation of conidial germination occurred after 12 h of inoculation. The globular appressoria of *M. anisopliae* were produced at the end of the germ-tubes after 24 h. Some germ-tubes penetrated the insect cuticle directly, without any detectable appressoria differentiation. Penetration was observed between 24 to 48 h after infection. About 48 h post infection, hyphal bodies and blastospores were observed in the haemolymph of live insect. Extensive tissue invasion and degradation over the next 24 h led to the death of the hosts within 72-96 h post inoculation. After death of the host larvae, hyphae re-emerged from the insect cadaver between 72 and 120 h. Subsequently, around 120 h, the whole body of the larvae was covered with a white mycelial layer. Conidiogenesis was observed on the insect cadaver between 120 and 144 h after inoculation.

B. Effect of repeated subculturing on surface properties of *M. anisopliae*- The commercial success of any mycoinsecticide depends significantly on the virulent nature of the infective propagules that are sprayed in the field. In this regard, the surface properties of the infective propagules form the basis of pathogen of adhesion and interaction with the host. Loss in virulence and lower CDE complex activities for *M. anisopliae* M34412 due to repeated sub-culturing on artificial media has been reported previously from our laboratory. In present study, in order to study the effect of repeated sub-culturing on conidial surface properties of *M. anisopliae* and its correlation with virulence against *H. armigera,* surface characteristics of conidia from 1st and 40th subculture were estimated.

Hydrophobic index measured using microbial adhesion to hydrocarbons (MATH) assay indicated that the surface of the 1st subculture conidia was more

hydrophobic than the 40th subculture, which was also corroborated by contact angle measurements. 1st subculture had faster sedimentation rate than 40th subculture conidia. Hydrophobicity and zeta potential measurements of *M. anisopliae* revealed the binding preferences of the fungal propagules. Atomic force microscopy (AFM) showed presence of more rodlet layers in aerial conidia of the 1st subculture than the 40th subculture indicating more roughness in 1st subculture conidia. The results were in parallel with the observation of more quantity of ~11 kDa and ~16 kDa proteins in aerial conidia of 1st subculture than 40th subculture. MALDI and LC-MS analysis identified these proteins as hydrophobins. Bioassays using *H. armigera* larvae with the 1st subculture conidia resulted in 97% mortality, whereas decreased mortality was observed with 40th subculture conidia indicating loss of virulence due to repeated subculturing. Hydrophobic interactions were predominant in the case of aerial conidia and are likely to be the most important factor in the host-pathogen interaction.

C. Degradation of cuticular hydrocarbons of *H. armigera* during *M. anisopliae* pathogenesis - In present study the ability of *M. anisoplae* to degrade cuticular hydrocarbons of the 3rd instar *H. armigera* larvae was observed. Hydrocarbon components consisted of a homologous series of *n*-alkanes (C_{14}-C_{33}), isomeric series of internally branched monomethyl alkanes, a series of internally branched dimethyl alkanes, tetra-methyl alkanes and alkenes. Within the *n*-alkane portion of the hydrocarbon profile in this species, odd-chain *n*-alkanes were quantitavely present in higher amounts, the predominant one being C_{27} (heptacosane). A gradual decrease in the amount of total hydrocarbons from 100% at 0 h to 21.8% at the end of 120 h was observed.

D. Production and surface properties of *M. anisopliae* blastospores- The highest production of blastospores was obtained in Admek medium (containing corn steep liquor) after 96 h. The 10% (v/v) mycelia inoculum was found to be optimum for the production of blastospores with yield of 1.2×10^8 blastospores/ml in 96 h at pH 5. It was observed that more than 90% of blastospores germinated after 6 h while *M. anisopliae* conidia required 16 h incubation to achieve more than 90% germination. No significant difference was observed in the cuticle degrading enzyme production of blastospores with conidia. The slower rate of sedimentation indicated the smooth and hydrophilic nature of blastospores surface as compared to that of conidia. Furthermore, HI and contact angle data confirmed the hydrophilic nature of

blastospores surface. Low root mean square (RMS) roughness values (13.4 nm) were measured by AFM for blastospores indicating a smoother surface as compared to that of conidia. The bundles of rodlet layers were not observed indicating the absence of hydrophobins on the surface of blastospores. Conidia were more robust for storage as they stayed viable (>95%) for than blastospores (<10% viability) after 2 weeks. The mortality of *H. armigera* was same with each of the *M. anisopliae* infective propagules. However, the LT_{50} was found to be lower with blastospores.

Chapter 5: Host range of *Metarhizium anisopliae* and its evaluation against *Aedes aegypti*- *M. anisopliae* was shown to be effective against a broad host range of insects belonging to the orders of Lepidoptera, Hemiptera, Coleoptera and Diptera. The biocontrol potential of the three *M. anisopliae viz*., M34412, M34311 and M81123 was evaluated against larvae and adult females of *Ae. aegypti* The strains resulted in 93 to 63% mortality as a result of fungal infection over the 8 d test period in case of adults and 98 to 10% mortality for larvae. LT_{50} varied between 3.36 and 5.76 d for treated adults, whilst control survival exceeded 28 d. Values of LC_{50} varied from 5.92×10^3 conidia/ml for M34412, 3.49×10^4 conidia/mL for M34311 and 5.12×10^5 conidia/ml for M81123. The most promising strains, *M. anisopliae* M34412, based on virulence and stability was used for lethal exposure time determinations. An exposure time of only 4 h was necessary to cause 50% mortality. Combination of the conidia with *M. verrucaria* CDE increased the larval mortality at lower conidial concentrations. These results suggest that *M. anisopliae* M34412 in combination with CDE could be promising biological control agents for use against larvae of *Ae. aegypti*.

Summary and conclusions- In conclusion, present investigations led to isolation of a highly virulent entomopathogenic fungal strain i.e. *M. anisopliae* M34412, better understanding of the pathogenesis of *M. anisopliae* and its interactions with the host insect. The study contributed significantly to the technology transfer and commercialization (currently in final phase) of *M. anisopliae* M34412.

Chapter 1

Introduction and Review of Literature

The population of the world estimated by the United States Census Bureau as of October 2012 is ~7.04 billion and is expected to be ~10.5 billion by year 2050. To satiate the need of all these people, approximately twice food production will be required by the year 2050 as opposed to what is produced now. However, the cultivable land available will be far less than today due to the encroachment of the growing population for living. Only solution for this is to increase productivity per hectare and minimizing crop and postharvest losses. One of the important hurdles in achieving the goal is the pest problem.

1.1 Pest

About fourteen percent of all crops are lost to insect destruction, resulting in economic losses estimated at $ 200 billion/year in the United States and $ 2 trillion worldwide (Pimentel, 2009). In case of India, the reported annual loss due to insect pests is around Rs. 8,63,884 million (Dhaliwal et al., 2010). Insect pests also have devastating effects on human health due to disease-transmitting insect vectors such as mosquitoes, biting flies, chiggers, fleas and ticks that spread infections to hundreds of millions of people each year. There are several insect pests which cause severe losses in different field crops, including *Helicoverpa armigera* on pulses, cotton, vegetables and sunflower, *Plutella xylostella* and *Spodoptera litura* on vegetables, woolly aphids (*Ceratovacuna lanigera*) on sugarcane, mealy bug (*Phenacoccus gossypiphilous* and *Maconellicoccus hirsutus*) on cotton and grapes, respectively (Table 1.1). From the pest mentioned in the Table 1.1, *H. armigera* is one of the important pests as it has a host range of more than 300 plant species including cotton, legumes, sunflower, wheat, sorghum, groundnut, field beans, tomato, tobacco, corn and a range of vegetables, fruit crops and tree species (Fitt, 1989; Rajapakse and Walter, 2007) (Fig. 1.1).

1.1.1 *Helicoverpa armigera*

Different species from genera *Helicoverpa*/*Heliothis* complex (*H. armigera, H. zea, H. virescens, H. exigua and H. punctigera*) feed on a wide range of hosts (Fitt, 1989; King, 1994; Matthews, 1999). The legume pod borer or cotton bollworm (*Helicoverpa armigera* Hübner) is a major constraint to crop production globally. It is

one of the most important insect pests in the world due to its mobility, high polyphagy, short life cycle and high reproductive rate (Lawo et al., 2008).

Table 1.1: Major insect pests of economically important crops

Host	Common name	Crops
Lepidoptera		
Helicoverpa armigera	American Bollworm	Maize, Sorghum, Sunflower, Cotton, Tobacco, Soybean, Pulses, Safflower, Rapeseed, Groundnut
Helicoverpa punctigera	Native budworm	Cotton, Sunflower, Lucerne, Soybean, Chickpea, Safflower
Heliothis virescens	Tobacco budworm	Tobacco, Cotton, Tomato, Sunflower, Soybean
Heliothis zea	Corn earworm	Maize, Sorghum, Cotton, Tomato, Sunflower, Soybean
Spodoptera litura	Rice cutworm	Rice, Chickpea, Soybean, Castor, Cotton, Tomato
Spodoptera exigua	Beet armyworm	Sugar beet, Tomato, Cotton
Cnaphalocrocis medinalis		
Marasmia patnalis	Leaffolder	Rice
Marasmia exigua		
Hemiptera		
Pyrilla perpusilla	Leafhopper	Sugarcane, wheat, maize, millet
Ceratovacuna lanigera	Wooly aphids	Sugarcane
Maconellicoccus hirsutus,	Mealy bug	Grapes, Rice
Brevennia rehi		
Dysdercus cingulatus	Red cotton bug	Cotton, okra, maize, oilseed crop
Dolycoris indicus Stal	Stink bug	
Piezodorus hybneri		Oil seed crop: Groundnut,
Piezodorus rubrofasciatus	Shield bug	soybean

Coleoptera		
Dicladispa armigera	Hispa	Rice
Phytoscaphus sp.	Leaf weevil	Soybean
Tanymecus indicus	Ghujia weevil	Wheat
Diptera		
Liriomyza cicerina	Leaf miner	Chickpea
Ophiomyia cicerivora	Gram stem miner	Chickpea
Asphondylia sp.	Gall fly	Linseed
Hydrellia sp.	Whorl maggot	Rice
Orseolia oryzae	Gall midge	
Dacus cucurbitae	Melon fruit fly	Melons

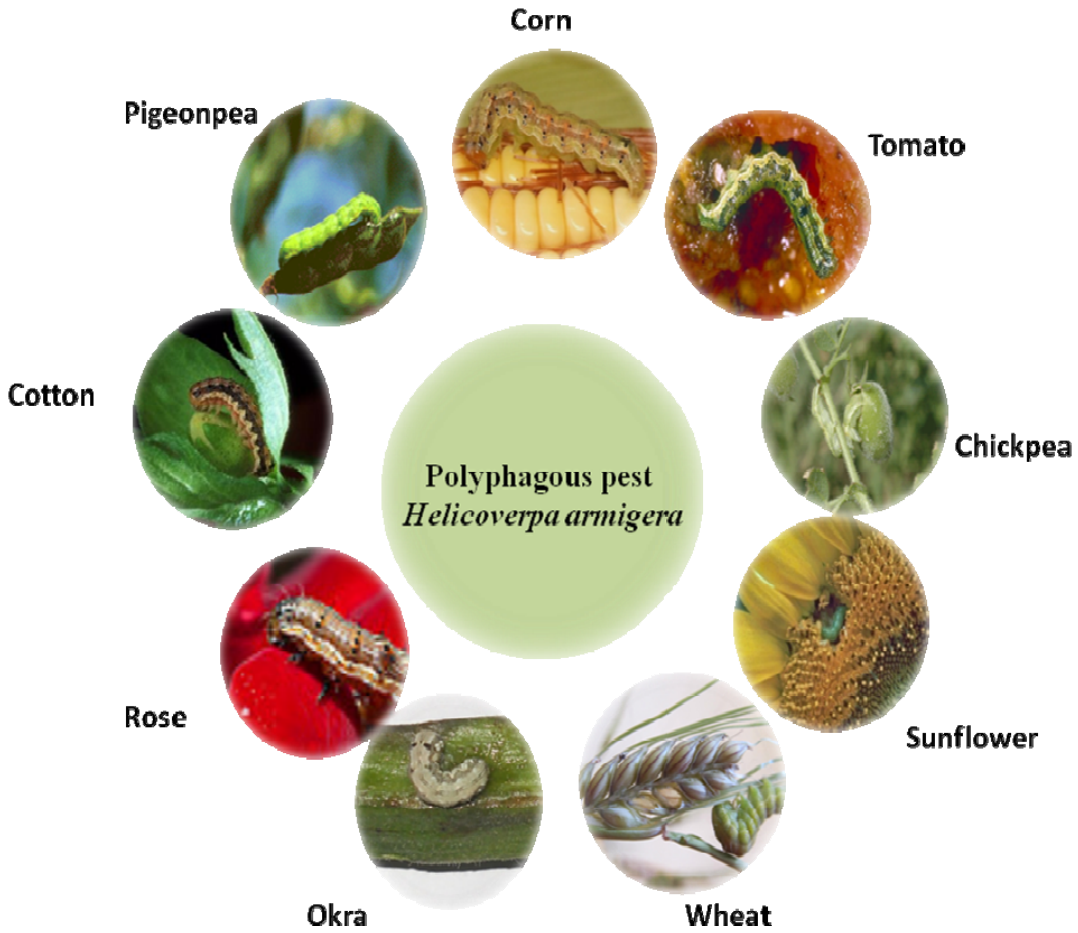


Fig. 1.1 Host range of *H. armigera*

Besides, the ability of ovipositing females to locate and utilize a wide range of hosts from a number of families is one of the major factors contributing to the pest status of this moth (Fitt, 1989; Zalucki et al., 1986). A wide range of wild plant species support larval development and important species in India include *Hibiscus sp., Acanthospermum sp., Datura sp., Gomphrena celesiodes* (Manjunath, 1989). *H. armigera* has a life span ranging 25-35 days. The females lay several hundred eggs on the lower surface of flowering and fruiting structures of the host crops. The eggs hatch after 3-4 days, while the larval stage lasts for 12-16 days. The larval period is divided into six instars of which the third and fourth instars are highly voracious and the most damaging ones. Monitoring of *H. armigera* populations is necessary to determine whether the threshold has been exceeded and control measures need to be undertaken. In cotton, a single larva per meter row causes significant economic loss (Khurana, 1997) and 2-3 larvae per plant can destroy all the cotton bolls within 15 days (Sharma et al., 2003). The caterpillars are aggressive, occasionally carnivorous and when the opportunity arises, cannibalistic. The pupa is a non feeding stage which lasts normally for 10-12 days and the moths remain alive for 4-5 days (Gowda and Sharma, 2005; Tamhane et al., 2005).

A conservative estimate was that over US $ 1 billion were spent on insecticides to control this pest and *H. armigera* had developed a high level of resistance to many of the commonly used insecticides (Kranthi et al., 2002).

1.2 Management of *H. armigera*

For the management of insect pests, a number of strategies are employed which include physical/mechanical control, cultural control, chemical control and biological control. For *H. armigera* management, these strategies have been used individually or in combination so as to maintain its population below economic threshold level (ETLs), as determined by the relationship between population density and economic loss.

1.2.1 Mechanical and cultural control

Mechanical control is the oldest method. It includes measures like a collection of egg masses and other inactive stages, removal of infested parts or whole plants, trenching, application of dry heat including exposure to sun rays during hot months to

reduce infestation by insects. This method is useful during the initial stage of pest incidence and when practiced as a concerted effort by a large number of farmers in a particular area. An example of mechanical control involves hand crushing the eggs of *Helicoverpa* on cotton in Egypt (Zethner, 1995). Farmers in Gulbarga district of Karnataka state and in Andhrapradesh, India, used indigenous method such as shaking off (*Helicoverpa* larvae) from the plants to manage the plant borer until chemical insecticides where introduced in early 1970's.

Numbers of cultural practices have been investigated to develop practical strategies so as to reduce the impact of *H. armigera* on different crops. The most studied practices include the use of short-season cultivars, adjusting the time of sowing (to avoid the peak incidence period), use of companion crops, trap crop. Other cultural practices, including deep ploughing after harvesting the crop (to expose the hiding and resting insects), plant spacing, higher soil moisture also reduce *H. armigera* infestation (Bergvinson, 2005).

Short season cultivars: Intercropping of pigeon pea with short season legumes such as soyabean or mungbean or sorghum reduces the influences of *H. armigera* on pigeonpea. Groundnut and coriander in chickpea were found to be beneficial to reducing the incidence of insect-pest. Chickpea intercropped with wheat, mustard, or safflower suffered less damaged by *H. armigera* (Rao et al., 2005).

Sowing time: Altering the sowing time of seeds reduces the impact of *H. armigera*. October sowing of chickpea resulted in greater grain yield in the Northn part of India due to lower *H. armigera* infestation. In case of pigeonpea, early sowing with the onset of monsoon rain helped in better crop establishment (Rao et al., 2005).

Trap crop: The trap cropping system has been developed to minimize the movement of *H. armigera* onto cotton; migration from cotton to other crops. Chickpea was used as trap crop in spring and pigeon pea in summer. Using okra, *Dolichos*, pigeonpea, sunflower, maize and marigold as trap crops for the management of *Helicoverpa* on tomato has shown promising result (Rao et al., 2005).

1.2.2 Chemical control

Chemical pesticides still remains the most effective practice to manage *Helicoverpa*, especially high value crops (cotton and vegetable). Significant reduction in larval population of *H. armigera* was observed by spraying insect growth inhibitors

such as diflubenzuron 150 to 170 g.a.i/ha (Kumar and Dahiya, 1997). Strip application of endosulfan (0.07%) reduced *Helicoverpa* damage and increased the grain yield. Application of pyrethroids on transgenic Bollgard cotton resulted in significantly lower percentage of *Helicoverpa* infestation and higher yield than untreated *Bt* cotton (Lanmbert et al., 1997). Effective management of *Helicoverpa* on cotton in Dharwad, Karnataka, India, through IPM, resulted in fewer pesticidial sprays and the cost: benefit ratio was 1:42.

Extensive application of pesticides during 1980's and 1990's, had contributed a lot to the heavy outbreaks of *H. armigera* (Ahmad et al., 2003). Despite the advantages of effectiveness, convenience, simplicity and flexibility, indiscriminate use of chemical pesticides in insect control programs has resulted in the development of resistance in insects to insecticides, the resurgence of pests, outbreak of secondary insect pests of several crops and change in pest status of many insects from minor to major category, destruction of natural enemies of pests and accumulation of pesticide residues in food and feed above the tolerance limit besides pollution of the environment.

In India, over 700 species of insect pests have developed resistance not only to individual chemicals but also to groups of chemicals, a notable example being *H. armigera* which has attained the status of a **'national pest'** (Bergvinson, 2005). Resistance has been reported in *H. armigera* to endosulfan, profenofos, thiodicarb, alpha-cypermethrin, cypermethrin, deltamethrin, lamda-cycalothrin, bifenthrin and cyfluthrin (Ahmad et al, 2003; Kranthi et al., 2001).

Therefore, it becomes imperative to search for alternative methods of control which are ecologically sound, reliable, economical and sustainable. Biological control offers a suitable alternative which includes the use of parasites, predators and microbial pathogens.

1.2.3 Biological control

Entomopathogenic bacteria, viruses, fungi, protozoa, nematodes, insects, birds and mammals have great potential as biocontrol agents of insect pests (Gopali and Lingappa, 2001; Jones et al., 2005; Malhi and Kaur, 2006; Rabindra and Ramanujam, 2007). Biological control involves large scale multiplication and liberation of such agents, or creating conditions under which the naturally occurring biocontrol agents

can act effectively. Biological control is an impotant component in *H. armigera* management and plays a vital role in sustainable crop production. The research on biological control of *Helicoverpa* received considerable impetus by the establishment of the Project Directorate of Biological Control (PDBC) in India. About 120 species of biological control agent have been recognized to be associated to *Helicoverpa* (Singh, 2007).

Parasites and predators: Insect parasites and predators are utilized in biological control programs through introduction, conservation and augmentation. Over 140 species of predators have been reported to directly prey on a specific life stage of *H. zea* or *H. virescens* (Kogan et al., 1989). Reduced feeding and movement on cotton was observed for these two species when parasitized by *Microplitis croceiepes* (Hopper and King, 1984). Use of *Trichogramma chilonis*, an egg parasitoid at 100,000/ha and *Chrysoperla carnea* at 50,000/ha 40 and 55 days after sowing was found to be effective in reducing populations of *H. armigera* and *Bemisia tabaci* populations in chickpeas (Kannaiyan, 2002).

Entomopathogenic bacteria: They are classified as spore formers and non-spore formers, crystalliferous and non-crystalliferous, obligate and facultative pathogens. Different genera of bacteria such as *Bacillus, Clostridium, Brevifaciens, Pseudomonas, Aerobacter, Cloaca, Proteus, Serratia* etc. have been identified as entomopathogenic forms. However, bacteria of only two genera from the order Eubacteriales including the genus *Bacillus* (Bacillaceae) and *Serratiae* (Enterobacteriaceae) are registered for insect control programs. *Bacillus thuringiensis Bt* var. *kurstaki*, *Bt*. var. *israelensis*, *Bt.* var. *sandiego*, *Bt*. var. *tenebrions*, *Bt.* var *entomocidus*, *Bt.* var *galleriae* and *Bt.* var *aizawai* have been employed for the control of various insect pests (Pawar, 2004; Tanweer et al., 1998). Though *B. thuringiensis* as a conventional insecticide and *Bt* transgenic crops are effective control measures for *Helicoverpa,* major drawback of *Bt*, however, is that the insecticidal crystal protein/toxin (*Cry1Ac*) acts only after ingestion by the insect. Secondly, repeated exposure of an insect population to *B. thuringiensis* induces the emergence of resistance (McGaughey, 1985).

Viruses: Insect-specific viruses can be very effective natural controls of several pests. Viruses belonging to Baculoviridae, Reaviridae, Iridoviridae, Poxviridae, Paroviridae, Picoviridae and Rhabdoviridae have been identified as entomopathogenic organisms

(Faukher and Boucias, 1985). There are more than 1600 different viruses that infect 1100 species of insects and mites. In India, work on insect viruses was initiated as early as 1968 with the report of nuclear polyhedrosis virus from *H. armigera* and *S. litura.* The nuclear polyhedrosis virus (NPV) of *H. armigera* has been studied extensively to evaluate its efficacy as a viral pesticide. HaNPV, a single embedded virion type has a high virulence against *H. armigera*. The virus, at a dose of 1.5-3×10^{12} polyhedra occlusion bodies (POB)/ha effectively controls *H. armigera* on crops. The successful commercialization of insect-pathogenic viruses has been limited. Viral pesticides are more expensive than chemical agents. Furthermore, many baculoviruses are host specific and thus cannot be used to control different pests. Besides, the action of baculoviruses on insect larvae is too slow to satisfy farmers. Also, viral preparations are not stable under the ultraviolet rays of the sun.

Fungi: Compared to other microorganisms, fungi are known to infect a broad range of insects including lepidopterans, hymenopterans, coleopterans and dipterans often resulting in natural epizootics. An added advantage in using entomopathogenic fungi is that infectivity is by contact and the action is through penetration (Deshpande, 1999). Therefore, fungi are the most important and widely used biocontrol agents. Being base of the present study, their role as biocontrol agents is described briefly in following section.

1.3 Fungi as biocontrol agents

Entomopathogenic fungi comprise a heterogeneous group of over 100 genera with approximately 750 species reported from different insects and living in diverse habitats including fresh water, soil surfaces and aerospaces (Hajek and St. Leger, 1994; Keller et al., 2003). Several of these entomopathogenic fungi with potential in pest management either belong to the Class Entomophthorales in the Zygomycota or the obsolete Class Hypomycetes in the Deuteromycota, now the asexually reproducing species of which are included in phyla Ascomycota.

Commonly encountered entomopathogens with broad host range are *Metarhizium*, *Beauveria*, *Nomuraea, Verticillium, Entomophaga* and *Paecilomyces* and they have been extensively studied for their efficacy as biocontrol agents (Butt et al., 1995, Charnley, 1989). *B. bassiana* has been identified in many insect species in

temperate and tropical regions and is used for pest control in Eastern Europe and China.

Table 1.2 Commercially produced entomopathogenic fungi

Fungus	Target pests	Product/Trade name	Company/Producer
Metarhizium anisopliae	Root weevils, Plant hoppers, Japanese beetle, Spittlebug and white grubs	Bio-magic	T. Stanes and Company Ltd. India
	Coconut beetles, White grub, Termites, leaf miners	Biomet Rich	Plantrich Chemicals and Biofertilizers Ltd, India
	Termites, Root grubs	Pacer	Agri Life, India
	Spittle bug; Sugarcane frog Hopper	Meta-Sin®	-
	Vine weevil, Scarab larvae on pasture, Spittle bugs, Cockroaches, Termites	BIO1020 Biogreen Bio-Path	Taensa, USA, Biocare Technology, Australia, Ecoscience, USA
Beauveria bassiana	Coffee berry borer, Cotton leaf roller, Root grubs, White flies, Aphids, Thrips	Bio-Guard-Rich	Plantrich Chemicals & Biofertilizers Ltd. India
	Caterpillars, Weevils, Leafhoppers, Bugs, Grubs, Leaf-feeding insects	Bio-power	T. Stanes and Company Ltd. India
	Helicoverpa	Racer	Agri Life, India
B. brongniartii (*B. tenella*)	Greenhouse whitefly thrips Mosquito larvae	*Beauveria* Schweizer	Lbu (formerly Eric Schweizer Seeds) Switzerland
	Betel	Arysta	Formerly NPP, Calliope France
Verticillium lecanii	Aphids; Coffee green bug; Greenhouse whitefly thrips	Vertalec	-
Hirsutella thompsonii	Citrus rust mite, Spittle bug; Sugarcane frog hopper	Mycar	-
	Coconut mite	Mycohit	Project Directorate of Biological Control, India
Culicinomyces clavisporus	Mosquito larvae	-	Austria, Belgium, Czech Republic
Paecilomyces fumosoroseus	Whitefly	PFR-97	ECO-tek, USA, PreFeRal
Lagenidium	Mosquitoes	*L. gigantium*	Registered in USA

(Compiled from Khan et al. 2012, Faria and Wraight, 2007, Shah and Pell, 2003; Strasser et al., 2000)

Beauveria bassiana based mycoinsecticide has been shown to be highly effective against coleopterans. *M. anisopliae* has been most extensively used for the control of spittlebugs on sugar cane in Brazil. *Verticillium lecanii* is a pathogen that has demonstrated better control of greenhouse pests, such as *Myzus persicae* (Sulcer) on Chrysanthemums. Commercially available fungal biocontrol agents and their target pests are given in Table 1.2.

The fungi are an important group of organism with a great variety of structural types. The vegetative growth forms are mycelia and unicellular yeast, whereas asexual spores such as conidia and sporangiospores are usually used as infective propagules in mycoinsecticide preparations. Sexually produced oospores, zygospores and resistant structures such as blastospores, arthrospores and chlamydospores have also been used in biocontrol formulations. Sometimes the fungal biomass can also be used. For example, *Lagenidium giganteum* biomass grown in a medium containing cottonseed flour, palm oil, cholesterol, lecithin, etc. can be used for mosquito control (Deshpande, 1999). In addition, certain fungal species are dimorphic in nature, i.e. exhibit two forms namely mycelium and yeast in liquid culture. In media containing high concentrations of nitrogen and carbon, the yeast-form cells eventually lead to the production of blastospores or hyphal bodies. Blastospores are hydrophilic, possess relatively few surface carbohydrates and do not survive after storage, but are the most virulent spore form. As several entomopathogenic fungi exhibit dimorphic growth it could be advantageous to grow organisms for blastospore production in liquid culture, for instance, *Paecilomyces fumosoroseus*, a biocontrol agent against sweet potato whitefly, can be grown in the form of blastospores that are resistant to drying. Short fermentation time could be one of the major economic advantages (Deshpande, 1999).

From all the reported entomopathogenic fungi, *Metarhizium anisopliae* (Metchnikoff) Sorokin is the second most widely exploited entomopathogenic fungus in biocontrol programs. It is known to infect over 200 species of insects. The present study is an attempt to understand the interaction between the widely used biocontrol agent i.e., *M. anisopliae* and a highly destructive insect host, *H. armigera.*

Metarhizium anisopliae (Metchnikoff) Sorokin

M. anisopliae (Metchnikoff) Sorokin was first isolated from the Coleopteran beetle, *Anisopliae austriaca* by Metchnikoff in 1878. It is ubiquitous in occurrence

with alternating life stages between a soil saprophytic stage and an insect pathogenic stage. The prominent taxonomic characters are the morphological features of the sporulating structures. The genus is defined on the basis of the arrangement of the phialides bearing chains and columns of dry and generally green, cylindrical or slightly ovoid conidia. The columns are formed by aggregation of the conidial chains. Under natural conditions, *Metarhizium* spp. produces two spore-types. Aerial conidia that are produced on specialized sporogenous hyphae, phialides during the saprophytic life stage or on host cadaver and are defined as asexual spores. Blastospores, the second spore-type with similar cell-wall characteristics to hyphae, are produced in the insect hemolymph.

M. anisopliae was previously included in phylum Deuteromycota (which is now obsolete and used only informally), Class Hypomycetes and Order Monoliales in its anamorphic state (Boucias and Pendland, 1998). Roy et al. (2006) assigned the perfect, sexual (teleomorphic) state of *M. anisopliae* to the division Ascomycota, class Sardariomycetes, order Hypocreales and family Clavicipitaceae. Further, a classification based on the size of conidia and host specificity was suggested by McCoy et al. (1988) - *M. anisopliae* var. *majus* with a conidial size of 18 µm and a host range restricted to scarabeid beetles and *M. anisopliae* var. *anisopliae* with 9 µm long conidia and a wide host range.

M. anisopliae var. *anisopliae* infects over 200 insect species (Zimmermann, 2007) across several insect orders including Coleoptera, Diptera, Hemiptera (including Homoptera), Hymenoptera, Isoptera, Orthoptera, Dermaptera and Lepidoptera (Schwarz, 1995). *M. anisopliae* as a species has a wider host range while certain strains and genotypes of *M. anisopliae* are more restricted (Ferron et al., 1972; Rombach et al., 1986; Bidochka and Small, 2005). Some genetic groups of *Metarhizium* from tropical and subtropical environments, especially strains of *M. anisopliae* var. *majus*, *M. flavoviride* and *M. album* show some insect-host preferences and are reported to be specific to Coleoptera, Orthoptera and Hemiptera, respectively (Rombach et al., 1986; Bidochka and Small, 2005). Species of *Oryctes* are only susceptible to all *Metarhizium* strains isolated from *Oryctes* species. On the other hand, isolates from soil were found to be highly virulent against specific pest insects, e.g. to the legume flower thrips, *Megalurothrips sjostedti* (Ekesi et al., 1998), or to the pod bug *Clavigralla tomentosicollis* (Ekesi et al., 1999). In arthropod hosts

of entomogenous fungi in Britain, Leatherdale (1970) listed *M. anisopliae* for two families of Coleoptera, i.e. Elateridae and Curculionidae (*Corymbites cupreus*, *Agriotes lineatus* or *A. obscurus, A. sputator*, *Sitona lepidus*) and on one Dipteran (*Lonchaea palposa*). *M. anisopliae* has also proven effective against mosquito larvae of the genus *Aedes*, *Culex* and *Anopheles* in the laboratory. Apart from the larval stage, eggs (*Aedes)* treated with *M. anisopliae* show a reduced hatch rate. Ovicidal property of *M. anisopliae* is best expressed at high humidity, which is a normal characteristic of anopheline oviposition sites (Bukhari et al., 2010)

Antagonistic properties of *M. anisopliae* have also been reported against some phytopathogenic fungi. The fungus was shown to be antagonistic to two strains of *Ophistoma ulmi* (*Ceratocystis ulmi*), the cause of Dutch elm disease (Gemma et al., 1984). Under greenhouse conditions and in the field, a strain of *M. anisopliae* was effectively used against *Phoma betae*, the blackleg of beet (Roberti et al., 1993). No phytotoxic effects on seedlings were observed, and *in vitro*, a clear inhibition zone between the two fungi was noted. Compatibility of *M. anisopliae* and other entomopathogenic fungi with the mycoparasites *Clonostachys* spp. (formerly *Gliocladium* spp.), *Trichoderma harzianum* and *Lecanicillium lecanii* was investigated by Krauss et al. (2004). Host-range tests showed that *M. anisopliae* was highly susceptible to all mycoparasites *in vitro* tests. However, co-application of mycoparasites with the entomopathogen did not affect their biocontrol efficacy *in vivo*.

Interactions between the biocontrol agent and the host insect is an important aspect in biocontrol and a better understanding will be useful in the development of effective biocontrol strategy for the management of different pests.

1.4 Fungus-insect interactions

Most of the entomopathogenic fungi have life cycles which synchronise with insect host stages and environmental conditions. In genera such as *Metarhizium, Beauveria, Nomuraea* and *Verticillium*, conidia are the means for spreading of infection in the host insect population. The infection process usually begins with contact of conidia onto the surface of the insect cuticle. For *M. anisopliae*, softer cuticular areas serve as entry points (Boucias and Pendland, 1998). Pathogenesis of the fungus in an insect host (mycosis) involves distinct steps - a) adhesion; b)

germination of conidia; c) penetration of the insect cuticle involving the breakdown of cuticular barriers by mechanical (appresorium formation) and enzymatic action (cuticle degrading enzymes); d) development of the fungus, i.e., proliferation of the fungus within the insect hemocoel eventually resulting in death of the insect due to different life stages within the host and toxin production and e) re-emergence of fungal hyphae on the insect cadaver (Fig. 1.2).

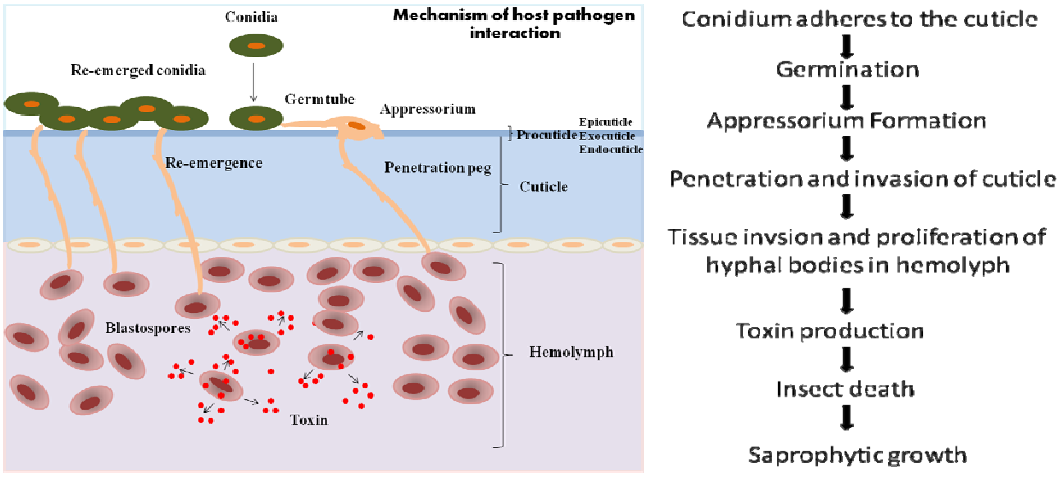


Fig. 1.2 Mechanism of fungal infection

In *M. anisopliae*, endoproteases are first secreted to disrupt proteins which expose the chitin to enzyme activity, specifically, lipases and esterases (St. Leger et al., 1987a). Enzyme activities are localized and are secreted in the vicinity of the germ tube and penetration plates. At this point during the penetration phase, an insect may resist infection by producing fungistatic phenols via the prophenol pathway (PPO), induced by cell wall constituents such as β-1-3 glucans, melanization, protease inhibitors or a physiological immune response from the hemolymph such as phagocytosis, nodulation or encapsulation (Charnley, 1989). Upon reaching the hemocoel, fungal hyphae can undergo changes to form a yeast-like phase. *M. anisopliae* produces toxins such as destruxins, which, along with blastospores, invade tissues resulting in death of the insect.

The entire process, is however dependent on factors such as host specificity, virulence of the fungus or the resistance of the host and finally the killing components (Yadav and Deshpande, 2010).

1.4.1 Specificity of fungus-insect interactions

Host specificity is evident during every step in the process of fungus-insect interactions, *viz.,* signal from the host, adhesion, formation of infection structures, penetration, production of killing components and vegetative growth in the haemocoel. Host specificity may also be related to the presence of fungistatic compounds (Butt et al., 1995; Sosa-Gomez et al., 1997). Nahar et al. (2008) reported the effect of repeated subculturing on the virulence of *M. anisopliae* against *H. armigera*. Usually, *in vitro* sub-culturing of entomopathogenic fungus affects viability and morphological, biochemical and molecular characteristics and most importantly, the virulence and host specificity too. Vandenberg and Cantone (2004) noted that the virulence of *Paecilomyces fumosoroseus* towards *Diuraphis noxia* or *Plutella xylostella* did not change even after 30 *in vitro* transfers. Interestingly, they further reported that different host passages had varying effects on virulence (Vandenberg and Cantone, 2004). For instance, the virulence of *P. fumosoroseus* towards *D. noxia* after 15 passages in *P. xylostella* decreased which did not regained even after five passages in *D. noxia*. Also, there was no change in the virulence of *P. fumosoroseus* towards insect hosts, *D. noxia* and *P. xylostella* after 15 passages in *D. noxia*. Xu et al. (2006) have compared the protease activity of a *Zoophthora radicans* strain that was highly infective toward *Pieris brassicae* (cabbage butterfly) larvae with that of isogenic strains that were adapted to *Plutella xylostella* (diamondback moth) larvae through serial passage. All strains produced three distinct serine proteases ranging from 25 to 37 kDa; however, the original strain from *P. brassicae* also produced large amounts of an approximately 46 kDa metalloprotease. It was reported that these proteases might be important for the infection process but might not be host specificity determinants (Yadav and Deshpande, 2010).

1.4.2 Fungal pathogenesis in the insect host

1.4.2.1 Conidia attachment/ adhesion

Entomopathogenic fungi display different strategies in their attachment to insect cuticle. The outermost layer of the insect cuticle, the epicuticle is relatively thin (1-2 μm) and composed of two layers - an outer layer composed of tanned proteins and polyphenols followed by layers of lipids bound to cuticulin. Beneath this is the

procuticle, approximately 200 μm thick and composed of chitin (Gordh and Headrick, 2011).

Infection propagules are either dry hydrophobic conidia as in case of *B. bassiana*, *M. anisopliae* and *N. rileyi*, or sticky hydrophilic conidia seen in *V. lecanii* and *H. thompsonii* (Sosa-Gomez et al., 1997). The adhesion of the infective stage may be divided into three phases: 1) **adsorption**, which is a passive phenomenon that is less host specific and mediated primarily by hydrophobic interactions between the conidial walls to the insect epicuticle and to a lesser extent electrostatic interactions; 2) **consolidation of the attachment**, which is more host specific and is an active process involving metabolic activity and 3) **germination and growth of the fungus** on the cuticle until penetration which is also an active process and also host-specific (Boucias and Pendland, 1991).

It has been reported that there is a conidial concentration dependent correlation between adhesion and mortality but not with host specificity. Since the hydrophobic conidia of entomopathogenic fungi bind in a nonspecific manner to the epicuticular surfaces of both susceptible and resistant hosts, it is difficult to demonstrate host specificity at the attachment stage. The presence of a fatty amide in the cuticle of a book lice, *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) decreases hydrophobicity and static charge leading to resistance to entomopathogenic fungi (Lord and Howard, 2004). The active host-specific adhesion is because of the lectin association of the conidial surface with the insect cuticle (Barranco-Florido et al., 2002). *M. anisopliae* aerial conidia are covered with rodlet layers, which has been suggested to be highly hydrophobic and an important factor for the adherence of aerial conidia to the insect cuticle (Sosa-Gomez et al., 1997). A water-insoluble layer of hydrophobin proteins is present on the surfaces of various fungal propagules and mediate attachment to hydrophobic surfaces (Sevim et al., 2012).

Hydrophobins: Hydrophobins are ubiquitous amphipathic proteins observed in the fungal kingdom with a broad spectrum of functions in fungal growth and development. They are reported to be involved in conidiogenesis and appressorium formation of *Magnapothe oryzae*, *M. anisopliae*, microsclerotia development in *Verticillium dahlia* and microconidia of *Fusarium verticillioides* (Talbot et al., 1993, 1996; Fuchs et al., 2004; Klimes and Dobinson, 2006; Li et al., 2006). Hydrophobins are also reported to play a role in spore dispersal of *Cladosporium fulvum*, aerial

hyphae formation of *Schizophyllum commune* (Wosten et al., 1994; Van Wetter et al. 1996; Whiteford and Spanu, 2001).

Hydrophobins are well characterized at the gene and protein levels. The role of hydrophobins in fungal growth and development were elucidated based on mutagenesis studies in fungi such as *Aspergillus fumigatus, A. nidulanse, Agaricus bisporus, Neurospora crassa, Ophiostoma ulmi, Schizophyllum commune, Magnaporthe grisea, Trichoderma harzianum, T. reesei and Claviceps pupurea* (Kershaw et al., 1998).

The distribution of the cysteines and the clustering of hydrophobic and hydrophilic residues allow hydrophobins to be grouped into two classes I and II. The aggregates formed by these two classes can be distinguished on the basis of their solubility and morphology. The assembled membrane formed by class I hydrophobins are highly insoluble (even resisting 2% SDS at 100°C) and can only be dissociated by high concentration of acids (such as formic acid or trifluoroacetic acid). Class II hydrophobins are less stable and can be readily dissolved by 60% ethanol or 2% SDS. Most of the class I hydrophobins form a microscopically identifiable rodlet structure outside the fungal cell wall (Asgeirsdattir et al., 1995, 1997). Class II hydrophobins form assemblies that lack distinct rodlet morphology. Despite these morphological differences, no obvious distinction between the functions of class I and class II hydrophobins within the fungal life cycle has yet emerged.

Hydrophobins are well characterized with eight conserved cysteine residues (Wosten, 2001). Class I and Class II hydrophobins can be distinguished on the basis of characteristic spacings between their cysteine residues (Wösten and Wessels, 1997) as depicted in Fig. 1.3. The cysteine pattern, however, is similar in both hydrophobin classes: the second and the third cysteine residue as well as the sixth and the seventh are located next to each other.

Class I X_{25-158}-C-X_{5-9}-C-C-X_{4-44}-C-X_{7-23}-C-X_{5-7}-C-C-X_{6-18}-C-X_{2-13}

Class II X_{17-165}-C-X_{7-10}-C-C-X_{11}-C-$X_{15/16}$-C-X_{6-9}-C-C-$X_{10/11}$-C-X_{3-8}

Fig. 1.3 Length of the amino acid sequences between eight cysteine residues in Class I and Class II hydrophobins. (X represents any amino acid other than cysteine and the subindex shows the number of amino acids present)

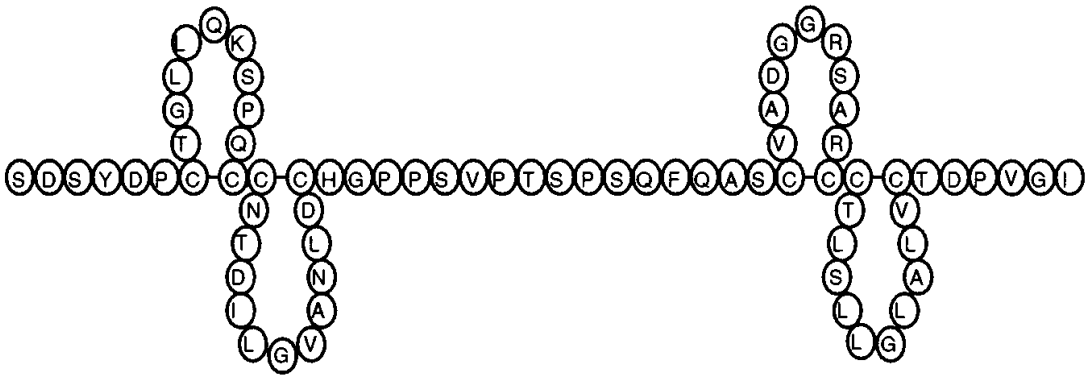


Fig. 1.4 Schematic representation of putative hydrophobin structures based on intramolecular disulphide linkages determined for *Ceratocystis ulmi* (originally constructed by Yaguchi et al., 1993)

The eight cysteine residues from class II hydrophobins are reported to be involved in four disulphide linkages resulting in formation of four loop-like structures (Kershaw et al., 1998). The cysteine linkages identified in *Ceratocystis ulmi* were between $Cys_{1\&2}$, $Cys_{3\&4}$, $Cys_{5\&6}$ and $Cys_{7\&8}$. Two of the four "loops" were composed predominantly of hydrophobic residues. Schematic representation of *Ceratocystis ulmi* hydrophobins in presented in Fig. 1.4 (Yaguchi et al., 1993). This characteristic order of cysteine residues distinguishes the fungal hydrophobins from other cysteine rich proteins.

In filamentous fungi, hydrophobins are critical components of the fungal conidial cell envelope, polymerizing to form bundles or fascicles containing rodlet-like structures (Fig. 1.5). Hydrophobins allow fungi to escape their aqueous environment, confer hydrophobicity to fungal surfaces in contact with air and mediate the attachment of hyphae to hydrophobic surfaces resulting in morphogenetic signals. The latter is important in initial steps of fungal pathogenesis where the fungus must attach to the hydrophobic surface of the host before penetration and infection can occur.

De Vries et al. (1999) first extensively studied SC3 hydrophobins from *Schizophyllum commune*. Recently, Kirkland and Keyhani (2011) demonstrated the expression and purification of *B. bassiana* hydrophobin (hyd2) from an *E. coli* host. Different hydrophobins reported from the fungi are summarized in Table 1.3.

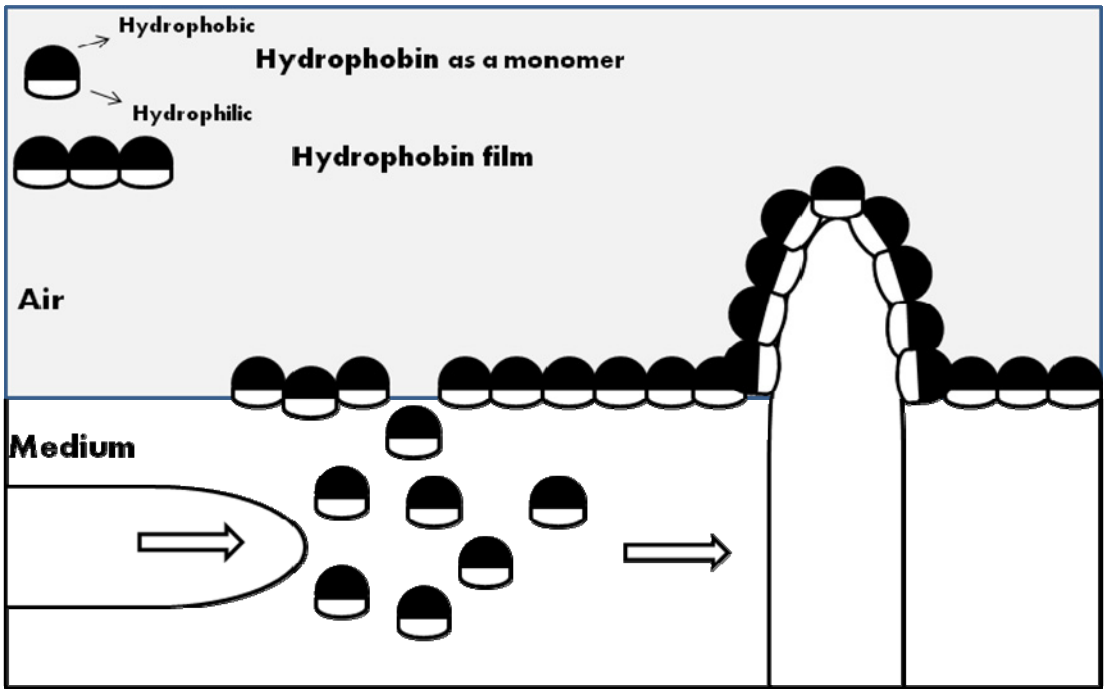


Fig. 1.5 Schematic model for formation of aerial hyphae in *M. anisopliae* (modified from Wessels, 1997; Wosten, 2001). Hydrophobic and hydrophilic parts of a hydrophobin molecule are indicated as black and white colours, respectively. The hydrophobin molecules secreted into the liquid culture medium form a hydrophobin film at the water-medium interface which decreases the surface tension of the liquid, allowing the hyphae to grow into the air. The rodlet-patterned hydrophobin film on the cell wall renders the aerial hyphae hydrophobic.

Table 1.3 Hydrophobins reported from fungi

Organism	Hydrophobin	Number of amino acid	M_r (kDa)	Reference or EMBL accession number
Class I Hydrophobins				
Ascomycetes				
Aspergillus fumigatus	RodB	140	14	Paris et al., 2003
Aspergillus nidulans	RodA	157	-	Stringer et al., 1991
Cladosporium fulvum	HCf-1	105	10	Spanu, 1997
Magnaporthe grisea	MPG	112	15	Talbot et al., 1993
Metarhizium anisopliae	SSGA	96	-	St. Leger et al., 1992
Neurospora crassa	EAS	108	8.2	Bell-Pedersen et al., 1992
Basidiomycete				
Agaricus bisporus	ABH3	119	9.1	Lugones et al., 1998
Dictyonema glabratum	DGH1	130	14	Trembley et al., 2002

Schizophyllum commune	SC1	109	13.5	Dons et al., 1984; Schuren and Wessels, 1990
Tircholoma terreum	Hyd1	108	23	Mankel et al., 2002; AY048578
Class II Hydrophobins				
Ascomycetes				
Claviceps fusiformis	CFTH1	394	36.5	De Vries et al., 1999
Fusarium verticillioides	Hyd4	100	-	Fuchs et al., 2004; AY155499
Magnaporthe grisea	MPH1,	102	-	Kim et al., 2001
Trichoderma harzianum	SRHI	89	-	Munaoz et al., 1997
Trichoderma reesei	HFBI	97	7.5	Nakari-Setala et al., 1996; Linder et al., 2001
Trichoderma viride	SRH1	89	-	DQ112069

1.4.2.2 Conidial germination

Optimum humidity for conidial germination and mycelial growth of entomopathogenic fungi is between 95-100%. However, conidia are able to obtain adequate moisture from the humid microenvironment of insect intersegmental membranes and thereby infect even in arid climates (Bateman et al., 1993). The surface characteristics of the host insect determine the availability of suitable nutrients which affect germination (St. Leger et al., 1992, 1994). Germination is also affected by the presence of fungistatic compounds (Butt et al., 1995; Sosa-Gomez et al., 1997). Blastospores are generally thought to have faster germination rates than conidia (Thomas et al., 1987). On the insect cuticle, conidia with faster germination rates may have a greater potential for infection by reducing loss due to desiccation, effects of other microorganisms or molting of the host (Al-Aidroos and Roberts, 1978; Al-Aidroos and Seifert, 1980; Charnley, 1984; Dillon and Charnley, 1985).

1.4.2.3 Penetration by mechanical (appresorium formation) and enzymatic action

Appressorium formation: Appressorium is one of the most important structures required for infection (St. Leger et al., 1994). St. Leger et al. (1991) proposed a model for the triggering of appressorium formation that involved tactile cues from the

surface of the insect cuticle. The model was supported by studies that have demonstrated *M. anisopliae* producing appressoria *in vitro* on hard hydrophobic surfaces (St. Leger et al., 1989; Nahar et al., 2008), and stimulated by low concentrations of complex nutrients (St. Leger et al., 1991). *In vivo* appressorium formation was also influenced by surface topography (St. Leger et al., 1991).

Production of Cuticle Degrading Enzymes: Apart from mechanical pressure by appresorium, penetration of the insect cuticle by entomopathogenic fungi also requires the production of cuticle degrading enzymes so as to degrade the highly refractory insect cuticle. *In vitro* experiments have shown that entomopathogenic fungi in submerged fermentation produce extracellular cuticle degrading enzymes mainly chitinases, proteases and lipases using insect cuticle as the sole carbon source (St. Leger et al., 1986). Extracellular enzymes appear sequentially in liquid culture media with *Metarhizium* and *Beauveria*. Proteolytic enzymes such as esterase, endopeptidase, aminopeptidase and carboxypeptidase are produced within the first 24 h of growth and *N*-acetylglucosaminidase appears later. Though chitin is the main structural component of the cuticle, endo-chitinase, which attacks the chitin polymer randomly, are produced in significant quantities after 4 days. Lipases are detectable after 5 days (St. Leger et al., 1989). St. Leger et al. (1986) assigned a major role to protease in cuticle degradation by *M. anisopliae* as chitinolytic enzymes appear after the enzymes of the proteolytic complex, which is in accordance with the cuticular structure wherein the proteins mask the chitin. Nahar et al. (2004) suggested that the constitutive production of chitin deacetylase (CDA) and chitosanase in *M. anisopliae* could be the primary step in the process of cuticle degradation by this fungus which shows low or delayed chitinase production. However, Fang et al. (2005) reported that the endochitinase from *M. anisopliae* show only marginal correlation with virulence towards the aphid *Myzus persicae,* while overproduction of endochitinase increased virulence of *B. bassiana* towards aphids. This variation could be attributed to the multiplicity of chitinases (Patil et al., 2000). The redundancy may ensure infectivity in the event that one system is inhibited (St. Leger et al., 1994).

1.4.2.4 Growth within the insect and toxin production

Growth of fungal pathogens in the hemolymph may be as yeast-like blastospores, hyphal bodies or protoplasts, rather than in the form of a mycelium.

These growth forms are designed to aid in dispersal through the insect hemolymph and evade the host immune system. For instance, avoiding phagocytosis by the host immune system may be attributed to the absence of certain carbohydrates and cell-wall structural components (e.g. chitin) in protoplast like conidia of *B. bassiana* that are produced *in vivo*, which are otherwise present on blastospores produced *in vitro* (Pendland et al., 1993).

The entomopathogenic fungi, *M. anisopliae*, *B. bassiana*, *B. brongniartii*, *P. fumosoroseus*, *V. lecanii*, *Tolypocladium* sp., and *H. thompsonii* produce different toxins such as destruxins, swainsinone, cytochalasin C, bassianin, beuvericin, oosporein, dipcolonic acid, enfrapeptins and hirsutellin A, B (Amiri-Besheli et al., 2000; Vey and Butt, 2001). Different insects vary in their susceptibility to these toxins. For example, lepidopteran insects are highly susceptible to cyclic depsipeptide destruxins produced by *M. anisopliae* (Clarkson and Charnley, 1996). Destruxins have been shown to be insecticidal by injection and, in some cases, when ingested through the oral cavity. Destruxins have diverse modes of action including depolarization of muscle membrane Ca^{2+} channels, inhibition of hemocyte function, and inhibition of vacuolar-type ATPase. In *M. anisopliae,* 19 variants of destruxins have been described while 17 have been described from *M. flavoviride*. Efrapeptins of *Tolypocladium* show insecticidal and miticidal effects against the potato beetle, bud-worm and diamondback moth and mites. Oosporein produced by *B. brongniartii* causes enzyme malfunctioning by redox reactions and is effective against cockchafer larvae. The hyphomycete, *H. thompsonii* produces an extracellular insecticidal protein hirsutellin A, which was shown to be effective against citrus rust mite, mosquito larvae, *Spodoptera frugiperda* and others (Vey and Butt, 2001).

1.4.2.5 Progression of the infection and host death

Once inside the hemocoel, the fungus proliferates and rapidly spreads throughout the body by means of the insect's open circulatory system. Hyphal bodies fill the hemocoel at the time of insect death with tissues being invaded and degraded to varying degrees and the nutrients become exhausted. Hyphae re-emerge from the insect cadaver and conidiate on the surface of the cadaver (Small and Bidochka, 2005).

The mechanism by which fungi invade and proliferate within the insect is dependent upon specific interactions occurring between individual hosts and pathogens. For instance, topical infection of the pyralid moth, *Lamprosema lateritialis*, with *B. bassiana* revealed that at least three days were required for germination and penetration of the integument prior to appearance in the body cavity. Within one week hyphal elements were present in the fat bodies, silk glands, malpighian tubules and gut, whereas, no infection of the muscles, nervous tissue or trachea was observed (Athuahene and Doppelreiter, 1982). Pekrul and Grula (1979) observed that in *B. bassiana* infections of *Heliothis zea* larvae, fat bodies became infected after 60-72 h post-infection. *Entomophaga grylli*, which proliferates within the insect by means of protoplast forms, infected the fat bodies and nervous system of the grasshopper. After two weeks, the fat bodies were extensively degraded and protoplasts were found attached to the gut, muscle and tracheae, although invasion of these tissues was not observed (Soper et al., 1983). Examination of the infection process by *B. bassiana* in the corn borer, *Ostrinia furnacalis*, has revealed that it proceeds through four stages with the hemolymph and lipid bodies being the first sites infected after cuticle penetration. This was followed by colonization of the alimentary tract, malpighian tubules, silk and reproductive glands and finally muscle, silk gland duct and nerve cord. The first indication of tissue damage and disintegration required at least 3-5 days with adult insects (Zhang et al., 1992). The fungus completed its life cycle on the insect within 6-7 days. In *Cornitermes cumulans*, greater amounts of mycelial extrusion points and conidiogenesis were observed in both insect legs and head region (Neves and Alves, 2000).

1.4.2.6 Insect immune response and resistance to infection

An understanding of fungal induced immune responses would help to identify the defence mechanisms of an insect and virulence factors of fungi required to overcome them. The fungal virulence determinants could be then manipulated to accelerate host death in a biological control scenario. The defensive arsenal of insects contains both passive structural barriers such as the cuticle and a cascade of active response to pathogens that gain access to the hemocoel. The active response includes melanisation, cellular reactions and humoral reaction to recognize the pathogen besides production of protease inhibitors.

Melanization: The oxidation of phenolic compounds to dihydroxyphenylalanine, typified by the production of brown or black melanin pigments is a common response of many insects to fungal infection. Melanin may partially shield the cuticle from enzymatic attack or may be toxic to fungi. However, such protection is incomplete and it has been suggested that melanization is primarily an effective defence against weak or slow growing pathogens, but ineffective against more virulent fungi (St. Leger et al., 1998).

Cellular reactions: Once the cuticle has been breached, the invading fungus is faced with the defence systems of the hemolymph. The responses to mycopathogens within the haemocoel include phagocytosis, encapsulation and nodulation. However, the fate of the fungal elements is uncertain. With the arbitrary injection method, Bidochka and Khachatourians (1987) found that haemocytes of the migratory grasshopper, *M. sanguinipes* encapsulate viable conidia of *B. bassiana*, however they fail to suppress conidial germination within the nodule. It was suggested that the production of toxins and extracellular proteases by *B. bassiana* could avoid encapsulation.

Humoral reactions: In response to fungal challenge, insects elicit an acquired humoral "immunity" to successive infection. Recognition of "non-self" is critical to the initiation of the hemocytic defence reaction and this selective response in insects depends on a specific chemical recognition on the part of the hemocytes. Serum and hemocyte cell membrane-bound lectins have been found in many insects (Mello et al., 1999). They probably play a role in immune defence reactions since they agglutinate pathogens as well as fungi (Mello et al., 1999). Thus, insect serum agglutinin may function as opsonin mediating the enhanced attachment of granulocytes to the hyphal bodies (Pendland et al., 1988).

1.5 Role of Epicuticular hydrocarbons during fungus insect interaction

The distribution of conidia on the insect cuticle is dependent on the cuticular lipids and the topography and also on the presence of setae and epicuticular folds (Sosa-Gomez et al., 1997). The epicuticle of insect is covered with a lipid-rich layer, usually composed of long-chain hydrocarbons (C_{21}-C_{35}) collectively with variable amounts of fatty alcohols, fatty acids and wax esters (Lockey, 1988; Pedrini et al., 2010). Cuticular free lipids consist mainly of aliphatic polar and non-polar compounds, which are extractable from the cuticle with organic solvents (Lockey,

1988). The primary function of epicuticular lipids is to set up a passive barrier to water evaporation through the cuticle (Hardley, 1994; Gibbs, 1998).

Entomopathogenic fungi have the capability to degrade insect cuticular lipids, particularly hydrocarbons and suggested that they were the components supporting fungal growth (Napolitano and Juairez, 1997). The first evidence of the complete catabolism of hydrocarbons by entomopathogenic fungi was obtained from *B. bassiana* and *M. anisopliae* (Crespo et al., 2002). In addition, alkane-grown *B. bassiana* showed enhanced virulence against *Acanthoscelides obtectus*, by increasing mortality from 22 to 60% after 14 d (Crespo et al., 2002).

Susceptibility or resistance of various insect species to fungal invasion may result from several factors, including differences in the structure and composition of the exoskeleton, the presence of antifungal compounds in the cuticle, as well as the efficiency of cellular and humoral defence reactions of invading insect (Vilcinskas et al., 1999). The cuticle composition strongly influences conidial germination, resulting in the differential susceptibility of various insect species to a fungal pathogen (Golebiowski et al., 2008). The nature of the inductive triggers has not been determined, but while the protein and chitin composition of the insect procuticle appears similar in all insects, the epicuticular components are heterogeneous and therefore have the potential to lead to different pathogen responses in particular insects. Cuticular fatty acids have a profound effect on conidial germination and differentiation: they are either toxic, fungistatic, or occasionally, for some pathogenic species, stimulatory (Golebiowski et al., 2008). Determination of the cuticular lipid profile is therefore of great significance in understanding the background of fungus-insect interction.

1.6 Objectives of the present investigations

Based on the above background, present study was aimed to contribute in studies on different aspects of host-pathogen interactions between *M. anisopliae* and *H. armigera* which may facilitate the development of better biocontrol strategies for the control of different insect pests. The objectives of the study were -

1. Screening of different *Metarhizium* isolates for the control of *H. armigera*.
2. *Metarhizium anisopliae* pathogenesis in *Helicoverpa armigera* and factors involved in their interaction:

A. Pathogenesis of *M. anisopliae* in *H. armigera*

B. Effect of repeated subculturing on surface properties of *M. anisopliae*

C. Degradation of cuticular hydrocarbons during pathogenesis of *M. anisopliae* in *H. armigera*

D. Production and surface properties of *M. anisopliae* blastospores

3. Host range of *Metarhizium anisopliae* and its evaluation against *Aedes aegypti*

CHAPTER 2

Materials and Methods

2.1 Materials

The materials and chemicals used in the studies were purchased from suppliers as shown in Table 2.1. All other chemicals used were of analytical grade, procured from S.d.fine-Chem Ltd., India; Sisco Research Laboratoies Ltd., India; Hi-media Laboratories, India; Loba Chemie, India.

Table 2.1 Source of chemicals and materials

Chemicals, Materials	**Source**
N-Acetylglucosamine (GlcNAc), Bovine serum albumin, chitin, chitosan, ethylene glycol chitin, glycol chitosan, glucosamine, gum arabic, laminarin , 3-methyl -2-benzothiazoline hydrazone (MBTH), *N*-acetyl glucosamine (GlcNAc), tyrosine, Alkane standard, Hexadecane	Sigma Chemical Co., USA
Hexane, Dicholomethane (DCM), Methanol, Hydroflouric acid (HF)	Merck Co.,USA
Pre-stained molecular weight markers for SDS PAGE	Bio-Rad, CA
Unicorn bags	Unicorn Imp and Mfg. Corp., USA

2.2 Media

The media used during the course of this work are listed in Table 2.2.

Table 2.2 Media

Name	**Constituents (g/l)**
Artificial Potato dextrose agar (PDA)	39.0 (Hi-media); pH 6.0
Natural Potato dextrose agar (PDA)	Peeled potato, 200; dextrose, 20; agar, 20; pH 6.0
Selective media	Peptone, 10; glucose, 20; agar, 18; streptomycin, 0.6; tetracycline, 0.05; cyclohexamide, 0.05; and dodine, 0.1 ml; pH 7
Yeast extract peptone glucose medium (YPG)	Yeast extract, 3.0; peptone, 5.0; glucose, 10.0; pH, 5.0-5.5

Chitin medium	KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract; 0.5; bacto-peptone, 0.5; chitin, 5.0; trace metal solution, 1ml (containing mg/ml: $FeSO_4$, 5.0; $MnSO_4$, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0); pH 6.0
Medium for blastospores production	Corn steep liquor 30, yeast extract 40, glucose 40; pH 5

2.3 Organisms and maintenance

All the *Metarhizium* isolates were maintained on artificial PDA. The stock cultures were maintained at 8ºC until use and also preserved by lyophilisation. Sub culturing was done every fifteen days and passage was done every three months on the host insect, *Helicoverpa armigera.* The culture was re-isolated after passage, maintained on PDA and used as a stock for all the experiments. *Myrothecium verrucaria* (MTCC 5191) was maintained on natural potato dextrose agar slants.

For repeated *in vitro* conidia transfers, *M. anisopliae* was grown on PDA. Conidia were harvested from PDA slants by scraping with a loop and sub-cultured to fresh PDA slants. This multi-spore *in vitro* transfer was repeated up to 40^{th} sub-culture.

2.4 Isolation of entomopathogenic fungi

Soil samples and infected insect samples were collected from various regions of Pune district, Maharashtra, India. Fifty-three strains were isolated from soil samples of different fields using a soil dilution method (Nahar et al., 2003), whereas 10 strains were isolated from insect hosts (greasy cut worm, mealybug, white grub, beetle, *Pyrilla perpussila*). The insects showing abnormal behaviour with poor coordination were collected from fields, kept at 28ºC until death and transferred to moist chambers for further mycosis and sporulation, if any. Conidia from sporulating cadavers were streaked on selective media and pure cultures were obtained by repeated subculturing (2-3 times) on the same media as described by Keller et al. (2003). After obtaining pure cultures, the isolates were maintained on a potato dextrose agar (PDA) slants for complete sporulation at 28ºC and 70-80% RH for 7

days. Following sporulation, the mother cultures were maintained at 8ºC until use. The numbering of the isolates was done with respect to the field number, plot number and sample number (Table 2.3).

Table 2.3 Nomenclature of *Metarhizium* isolates

Isolate No.	Nomenclature	Field No.	Plot No.	Soil sample No.	Isolate No.	Crop in the field/insect collected
M1	M1311	1	3	1	1	Tomato
M10	M34210	3	4	2	10	Custard apple
M33	M101133	10	1	1	33	Brinjal
M54	M16154	16	1	-	54	Sugarcane mealybug
M63	M161063	16	10	-	63	Sugarcane-*Pyrilla perpussila*

2.5 Conidial germination and appresorium formation

For germination, the conidial suspensions (1×10^7 conidia/ml) of 1st and 40th subcultures and blastospores of *M. anisopliae* were prepared in 0.1% (w/v) Tween 80 as described earlier (Nahar et al., 2003). The suspensions were spread on PDA agar on slides and incubated at 28°C and 70-80% RH for 24 h. Germ tube formation was observed under a light microscope every 2 h. For each sample, the germinated and non-germinated conidia were counted in 10 different fields, in triplicate.

Formation of appresoria on artificial surfaces was checked as described by Xavier-Santos et al. (1999). Briefly, the conidial suspensions of 1st and 40th subcultures and blastospores (100 µl of 1×10^7 cfu/ml) of *M. anisopliae* prepared in 0.1% (w/v) Tween 80 were inoculated separately in 5 ml YPG liquid medium and incubated at 28°C with 70-80% RH. Initiation of germination was observed microscopically at 2 h intervals. Upon initiation of germination, the conidia were separated from the YPG medium by centrifugation at 10,000 g for 10 min.

Germinated conidia were washed twice with sterile distilled water and resuspended in 100 µl sterile distilled water. The suspensions of germinated conidia were placed separately at the centre of polypropylene petri plates, which were sealed with parafilm and incubated at 28°C with 70-80 % RH. The plates were observed periodically under the microscope for development of appresoria during the next 24 h. The number of conidia producing germ tubes and/ or appresoria was counted in 10 fields for each plate and each experiment was carried out in triplicate. In total, for each plate, no less than 300 conidia were recorded as germinated or non-germinated.

2.6 Production of infective propagules

2.6.1 Production of conidia

Conidia of *M. anisopliae* M34311, M34412 and M81123 were produced by solid-state fermentation. The inoculum was prepared by inoculating 2×10^7 conidia in 200 ml YPG medium and incubating at 28ºC under shaking condition (180 rpm) for 2 days. The unicorn-bags (autoclavable, type/14 with single microvented filter of 0.2 mm, 2 kg capacity, 64×36 cm, Unicorn Imp & Mfg Corp, USA) filled with 2 kg of rice as a substrate were autoclaved at 121ºC for 45 min. and inoculated with the 200 ml of mycelial inoculum (mycelial biomass, 35 g±3 g wet weight). The bags were incubated at 28ºC and 70-80% RH for 14 days, after which they were dried at 37ºC for 2 days to bring the moisture <20%. The conidia were harvested with a Mycoharvester (CABI Bioscience, UK) and the yield (g/kg substrate) and percent viability of first quality conidia (mesh size < 100 µm) were determined from three different bags for each isolate. For determining percent viability, conidia suspensions were prepared in 0.1% (w/v) Tween 80 and the count was adjusted to 1×10^2 conidia/ml for inoculating PDA plates in triplicate for each isolate. The plates were incubated at 28ºC and 70-80% RH for 72 h and colonies were counted. The experiment was conducted in triplicate.

2.6.2 Production of blastospores

Conidial suspension was prepared by scraping from well sporulated 7 d old fresh slant culture of *M. anisopliae* into sterile 0.1% Tween-80 (w/v). To prepare a

primary culture, 1 ml of a suspension of conidia (1×10^7 conidia/ml) was inoculated into 100 ml of YPG medium in 250 ml Erlenmeyer flask and cultured at 28ºC on a rotator shaker at 180 rpm for 48 h.

Table 2.4 Composition of different media used for blastospores production

Mediums	**pH**	**Constituents (%, g/100ml)**
YPG medium	6.6	Glucose, 1; Yeast extract, 0.3; Peptone, 0.5
Jackson medium	4.8	KH_2PO_4 , 0.2; $MgSO_4.7H_20$, 0.03; $FeSO_4$, 0.005; $MnSO_4$ 0.0015;$ZnSO_4.H_2O$ 0.0014, $CoCl_2.6H_20$ 0.0036; $CaCl_20.04$; Casamino acids 1.32; Glucose 8
Paris medium	4	KH_2PO_4 , 0.036; $MgSO_4.7H_20$, 0.060; $Na_2HPO_4.12H_2O$, 0.142, KCl 0.1; NH_4NO_3, 0.07; Glucose, 8
Kondryatiev medium		KH_2PO_4 , 0.2; $MgSO_4.7H_20$, 0.2; $CaCl_2$, 1; $NaNO_3$, 0.5; Maltose, 2
Admek (CSL containing medium)	5	CSL, 3; Glucose, 4; Yeast extract, 4
CSS containing medium	5.2	CSS, 3; Glucose, 4; Yeast extract, 4
CSL containing Molases medium	5	Molasses, 3; Glucose, 4 Yeast extract, 4
Czapek-dox medium	5	$MgSO_4.7H_20$, 0.1;$FeSO_4$, 0.05; $MnSO_4$, 0.001; $NaNO_3$, 0.2; Sucrose, 3

For blastospore production, different media mentioned in Table 2.4 were used and based on the blastospore concentration, medium with corn steep liquor was used for subsequent production. *M. anisopliae* was grown in 200-ml corn steep liquor containing medium in 1000 ml baffled Erlenmeyer flasks under shaking condition (180 rpm) and incubated at 28ºC. Blastospore inocula for shake flask studies were obtained by inoculating blastospore production media with secondary inoculums 10 % mycelia of 48 h old *M. anisopliae*. These shake flask cultures were grown at 28ºC and 180 rpm in a rotary shaker incubator. After 4 days growth, shake flask cultures were filtered through autoclaved muslin cloth to remove mycelial fragments. The filtrate was centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant was

used to determine the pH and then discarded. The resultant blastospores pellet was washed with 10 ml of sterile distilled water and re-centrifuged. The water was then decanted and the blastospores were used for further studies.

2.7 Conidial settling time

The conidia settling rates for *M. anisopliae* isolates M34311, M81123 and M34412 (1^{st} subculture, 40^{th} subculture and blastospores) were determined as described by Jeffs and Khachatourians (1997). Conidia suspensions (1×10^7 conidia/ml in 0.1 % (w/v) Tween 80) were prepared to obtain an initial absorbance of 0.600 at 540 nm. The cuvettes were allowed to stand for 6 h and the absorbance was recorded at hourly intervals. The experiment was repeated thrice using freshly prepared conidial suspensions and the settling rate was expressed in percent (ST_{50}).

2.8 Adhesion to polystyrene

This test is based on adherence of hydrophobic surface on polystyrene (Rosenberg, 1981). One ml volume of a fixed concentration of the 1^{st} and 40^{th} subculture *M. anisopliae* M34412 conidial suspensions in 0.1 M potassium phosphate buffer (at pH 3.0, 5.0 and 7.0) was poured into polystyrene petri plates and left to settle for 24 h. The supernatant was then removed by the insertion in a beaker with 1.5 L deionized water and agitated at 1000 rpm. After 2 h, the dish was observed under a microscope to quantify the adhesion of cells using microscopic images.

2.9 Microbial adhesion to hydrocarbons (MATH) assay

Hydrophobicity of the conidial surface was determined by the method of Smith et al. (1998). Briefly, aerial conidia of the 1^{st} and 40^{th} subculture as well as blastospores of *M. anisopliae* M34412 were washed in PUM buffer (g/L: 22.2 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4.7H_2O$, pH 7.1). Conidial suspensions were adjusted to 0.4 OD (A_{470}) and dispensed with (3 ml) of PUM buffer into acid-washed glass tubes. Hexadecane (300 µl) was then added to each tube and the tubes were vortexed three times for 30 s each. The vortexed tubes were allowed to stand at room temperature for 15 min. and the non-polar hexadecane phase was carefully removed and discarded. Tubes were then cooled to 5ºC and any residual hexadecane removed. The tubes were then returned to room temperature and

absorbance of the resultant conidial suspensions was determined at 470 nm. The hydrophobic index was calculated using the following equation:

$$\frac{(A_{470\text{control}} - A_{470,\ \text{hexadecane treated}})}{(A_{470,\ \text{control}})}$$

2.10 Contact angle measurement

Contact angle measurements of the *M. anisopliae* M34412 were performed on a GBX model (DIGIDROP contact angle instrument) using Windrop software. The 1st and 40th subculture was grown on PDA slants till sporulation was observed. Pieces of the culture mat of 0.5 cm × 0.5 cm were cut out of the slants and placed on glass coverslips. For blastospores, 1×10^8 blastospores/ml was spread on small pieces of glass coverslip. A 10 µl drop of the suspension (sterile dH_2O) was placed onto the surface of the culture mat and contact angle was determined. All contact angle measurements were carried out at room temperature (27^oC) and constant humidity (40-50%) with a standard deviation of $\pm 2^o$. Extreme care was taken in carrying out these measurements to monitor contact angle values within 1 min to avoid evaporation effects. Five separate observations were averaged to obtain one representative value of contact angle for each studied surface and liquid.

2.11 Zeta potential measurement

Conidia of 1st and 40th subculture and blastospores of *M. anisopliae* M34412 were suspended in 2 ml Tween-80 (0.1%, pH 6.3) to get concentration of $1x10^6$ cfu/ml. For each sample, three Zeta potential measurements (with five runs) were taken using a particle size analyzer (Brookhaven Instrument Corporation, USA).

2.12 Production of cuticle degrading enzymes

The extracellular production of induced CDE's, i.e., chitinase (EC 3.2.1.14), chitosanase (EC 3.2.1.132), protease (EC 3.4.21.62) and lipase (EC 3.1.1.3) for the 3 isolates of *M. anisopliae* (M34412, M34311 and M81123), blastospores of *M. anisopliae* M34412 and *M. verrucaria* MTCC5191 was studied in chitin containing medium as described by Vyas and Deshpande (1989). The constitutive production of extracellular CDA (EC 3.5.1.41) was checked in YPG medium. For enzyme

production, 500 µL of 1×10^7 cfu/ml were inoculated in 50 ml of YPG and chitin medium and the flasks were incubated at 28°C for 72 and 96 h, respectively.

For CDE complex used in bioassay, *M. verrucaria* MTCC 5191 was grown for 7 d in chitin containing medium. The culture supernatant was collected by centrifugation (5000 g for 10 min.), lyophilized and stored at -20ºC until use.

2.12.1 Chitinase assay

Total chitinase activity in the culture filtrate was estimated colorimetrically using acid-swollen chitin as a substrate. To prepare acid-swollen chitin, 10 g chitin (purified powder from Crab shells, Sigma) was suspended in 300 ml *o*-phosphoric acid (88% w/v) and left at 4-8ºC for 1 h with occasional stirring. The mixture was poured into ice-cold distilled water (4 L) and left for 30 min. The swollen chitin was repeatedly washed with ice-cold distilled water, followed by a wash with 1% (w/v) $NaHCO_3$ solution to adjust the pH to 7. The swollen chitin was then dialysed at 4-8ºC against distilled water. After homogenisation in a waring blender for 1 min., the concentration of acid swollen chitin was adjusted to 7 mg/ml by adding 50 mM acetate buffer, pH 5.

The reaction mixture containing 1 ml 0.7% acid swollen chitin, 1 ml 50 mM acetate buffer, pH 5 and 1 ml of culture filtrate was incubated at 50ºC for 1 h. After incubation, the reaction mixture was centrifuged at 10,000 rpm for 10 min. and 0.5 ml of the supernatant was used for the estimation of *N*-acetylglucosamine. The supernatant (0.5 ml) was added to 100 µl borate buffer (pH 9.2, 0.02M). The reaction mixture was kept in boiling water bath for 3 min., cooled under running tap water and 3 ml of *p*-dimethyl amino benzaldehyde (DMAB) reagent was added. The reaction mixture was mixed properly and incubated at 37ºC for 20 min. After incubation, the *N*-acetylglucosamine (GlcNAc) residues produced were estimated colorimetrically at 585 nm (Reissig et al., 1955). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of GlcNAc per min.

2.12.2 Chitosanase assay

Chitosanase activity was estimated using acid-swollen chitosan as a substrate. For preparation of acid-swollen chitosan, crystalline chitosan (Sigma) was swollen

with 10 N HCl. The pH of the swollen chitosan was adjusted to 7 with 1 N NaOH. Repeated washing of swollen chitosan was carried out with ice-cold distilled water by centrifugation at 10,000 g for 10 min. The swollen chitosan was dialysed at 4-8ºC against distilled water. After homogenisation in a Waring blender for 1 min., the concentration of swollen chitosan was adjusted to 10 mg/ml by adding 50 mM acetate buffer, pH 5.

The assay mixture containing 1 ml acid-swollen chitosan (10 mg/ml), 1 ml of 50 mM acetate buffer, pH 5, and 1 ml of enzyme (culture filtrate) was incubated at 50ºC for 1 h. After incubation the reaction mixture was centrifuged at 10,000 rpm for 10 min. and 1 ml of the supernatant was used for the estimation of glucosamine. The amount of glucosamine produced was determined using the method of Reissig et al. (1955). Supernatant, 1.0 ml, was added to 0.2 ml acetic anhydride (5 % in acetone) followed by addition of 1 ml borate buffer (pH 9.2, 0.02 M). This reaction mixture was kept in a boiling water-bath for 3 min. and subsequently cooled under running tap water. DMAB reagent (7.8 ml) was added to the reaction mixture and then incubated at 37ºC for 20 min. after mixing. The absorbance was measured at 585 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of glucosamine per min.

2.12.3 Chitin deacetylase assay (CDA)

CDA activity was measured using acetylated ethylene glycol chitosan as a substrate which was prepared according to the method of Araki and Ito (1975). For preparation of the substrate, ethylene glycol chitosan (40 mg) was treated at 4ºC with 400 mg of $NaHCO_3$ and 200 µmol of acetic anhydride in a total volume of 4.5 ml and kept at 4ºC. After 24 h, 200 µl of acetic anhydride was added and the mixture was allowed to stand for further 24 h at 4ºC. After thorough dialysis, the product, acetylated ethylene glycol chitosan (1 mg/ml) was used as a substrate for the assay of CDA.

The assay for CDA was carried out as described by Kauss and Bausch (1988). The reaction mixture containing 100 µl of 50 mM sodium tetraborate buffer, pH 8.5, 100 µl of 1 mg/ml acetylated ethylene glycol chitosan, and 50 µl enzyme (culture filtrate) was incubated at 37ºC for 30 min. The reaction was terminated with the addition of 250 µl of 5% (w/v) $KHSO_4$. For colour development, 250 ml of 5% (w/v)

$NaNO_2$ was added and allowed to stand for 15 min., and then 250 µl of 12.5% (w/v) ammonium sulfamate ($N_2H_6SO_3$) was added. After 5 min., 250 µl freshly prepared 0.5% (w/v) 3-methyl-2-benzothiazoline hydrazone (MBTH) was added and the mixture was heated in a boiling water bath for 3 min. The tubes were cooled under tap water and 250 µl of freshly prepared 0.5% (w/v) $FeCl_3$ was added. CDA activity was estimated spectrophotometrically at 650 nm. One unit of enzyme released 1 µmol of glucosamine from acetylated ethylene glycol chitosan per min.

2.12.4 Protease assay

Protease activity was measured using Hammerstein casein as a substrate (Nahar et al., 2008). The reaction mixture contained 100 µl of enzyme solution, 1 ml Hammerstein casein (1%) and 9.9 ml of 200 mM carbonate- bicarbonate buffer, pH 9.7. Enzyme reaction was carried out at 35ºC for 20 min. and terminated by the addition of 3 ml trichloroacetic acid (TCA) (2.6 ml of 5% TCA - 0.4 ml of 3.3 N HCl). The absorbance of the TCA soluble fraction was measured at 280 nm. One unit of enzyme liberated 1 µmol of tyrosine per min.

2.12.5 Lipase assay

Lipase activity was determined as described by Pignede et al. (2000). The substrate emulsion was prepared with olive oil (20 ml) and gum arabic (165 ml, 10%, w/v, Sigma). The reaction mixture contained 1 ml enzyme, 5 ml substrate emulsion and 2 ml of 50 mM phosphate buffer at pH 6.8. It was incubated at 37ºC for 1 h under shaking condition (80 rpm). The reaction was stopped with 4 ml of acetone-ethanol (1:1) containing 0.09% phenolphthalein as an indicator. Enzyme activity was determined by titration of the fatty acids released with 50 mM NaOH. One unit of lipase was defined as the amount of enzyme that released 1 µmol of fatty acids per min.

2.13 Protein estimation

Protein was estimated according to Lowry et al., (1951) using bovine serum albumin as the standard.

2.14 Protein identification

2.14.1 Protein extraction, estimation and analysis by SDS-polyacrylamide gel electrophoresis

The rodlet layers were separated from the conidia surface by incubating dry conidia with 48% hydrofluoric acid for 72 h at 4ºC. The contents were centrifuged (9000 g, 10 min.) and lyophilised under vacuum. The lyophilised material was reconstituted in 100 mM Tris buffer (pH 5.8), dialysed overnight with 20 mM Tris buffer (pH 5.8) and protein was estimated by Lowry's method. An aliquot was subjected to SDS-PAGE (15% gel) at pH 8 according to Laemmli (1970) and visualized by comassie blue staining following standard protocols and subjected to further analysis.

2.14.2 Analysis of rodlet hydrophobins by mass spectrometry (Q-TOF-MALDI-TOF-MS/MS and nano-UPLC Q-TOF MS-MS)

Protein bands on SDS-PAGE (15% gel) were excised manually from the gel and completely destained by washing in 50% acetonitrile (ACN)/50% mM ammonium bicarbonate followed by dehydration with 100% ACN so that the gel piece appear shrunken and white. The gel piece was reduced in 10 mM DTT (in 100 mM ammonium bicarbonate) for 60 min. at 56ºC. Alkylation was done by incubating with 55 mM iodoacetamide (in 100 mM ammonium bicarbonate) for 45 min. in dark at room temperature (RT). The gel piece was washed again and dehydrated with ammonium bicarbonate and ACN as described earlier. Subsequently, gel trypsin digestion was carried out overnight with 0.5 µg of trypsin (Porcine trypsin, Promega) in 20 µl of 50 mM ammonium bicarbonate at 37ºC. The digested solution was collected in separate tubes. Peptides were extracted with 50 µl extraction buffer (50% ACN/2% formic acid) by vortexing and sonication and the supernatant was combined with the initial digested solution. The extraction step was repeated twice. The 120 µl solution containing the peptide solution was then vacuum dried.

Extracted peptides were analysed by Q-TOF-MALDI-TOF-MS/MS as well as by nano-UPLC Q-TOF MS-MS (SYNAPT High Defination Mass spectrometer, Waters Corporation, Milford MA, USA). Mass spectral acquisition was carried out by MALDI survey method and Data independent acquisition (MS^E) method. Protein Lynx Global server version (PLGS) 2.4 software (Waters) was used for data processing and database searches. The MS/MS data were searched against the fungal

hydrophobin database constructed separately with the following parameters: peptide tolerance of 20 ppm, fragment tolerance of 0.05 D, one missed cleavage, carbamidomethylation of cysteines and possible oxidation of Methionine.

2.15 Analysis of Hydrocarbons

2.15.1 Extraction of cuticular hydrocarbons from infected insect

Hydrocarbons were extracted from the infected larvae by immersing in hexane (1 g: 10 ml) for a period of 24 h. The extract was partitioned against distilled water (5:1, v/v). The layers were separated and the solvent was concentrated under a gentle stream of nitrogen. The concentrated extract was loaded onto a mini column of silica gel (100-200 mesh, 75-150 µm, pore size- 25Å, Sigma-Aldrich Co., St. Louis, USA) and hydrocarbons were extracted with n-hexane (10 ml). The eluted fractions were concentrated under stream of N_2 and resuspended in 1 ml hexane containing 100 ng/µl of the internal standered *n*-bromodecane.

2.15.2 GC and GC-MS analysis

GC analysis was done on a Varian CP3800 using a CP-Sil 8CB column with nitrogen as a carrier gas. The injector and detector temperature were at 300ºC and 310ºC respectively. The oven temperature was programmed as follows: 100ºC, hold for 2 min.; 100-220ºC @ 10ºC/min.; 220-325ºC @ 5 ºC/min. and hold at 325ºC for 10 min. Identification of individual components was done by GC-MS on a Agilent 7890B GC equipped with an Agilent 5975C MSD. The MS source temp was 230ºC and the oven temperature was as mentioned above. Individual hydrocarbon was identified by comparison of their mass spectra with those of authentic standards.

2.16 Microscopy

2.16.1 Light microscopy

Fungal germination was observed under a light microscope (Leitz Laborlux S, Germany). Hyphal growths, indication of fungal infections on cadaver of insects after 3-4 d were observed visually and using stereo-microscope.

2.16.2 Scanning electron microscopy (SEM)

Larvae and conidia were fixed overnight at 4ºC with 0.1 M glutaraldehyde in sodium cacodylate buffer, pH 7. The specimens were washed thrice in sodium cacodylate buffer at room temperature and fixed in 0.5% osmium tetroxide in sodium cacodylate buffer for 4 h. Subsequently, they were rinsed in sodium cacodylate buffer, dehydrated in a series of acetone solutions (30-100%). The specimens were air dried, mounted on stubs and sputter-coated with gold (5 nm) and viewed under a FEI Quanta 3D SEM at 10 KV.

2.16.3 Transmission electron microscopy (TEM)

For transmission electron microscopy, *M. anisopliae* conidia were kept overnight in 2.5% glutaraldehyde fixative in 0.1 M sodium cacodylate buffer (pH 7) at 4°C. The conidia were washed thrice in sodium cacodylate buffer at room temperature. The specimens were dehydrated in a series of acetone (30-100%), and then put on TEM carbon coated grids, dried overnight and examined with an electron microscope (Tecnai 20 G2, operating at 200 KV).

2.16.4 Atomic force microscopy (AFM)

M. anisopliae M34412 conidia (1^{st} and 40^{th} subculture) and blastospores were harvested from PDA slant and liquid medium, respectively. Blastospores were washed twice with distilled water. Conidia and blastospores were suspended in 0.1% Tween-80 and distilled water, respectively. Air-immobilisation technique was used to fix the conidia and blastospores on cover slip.

Atomic force micrographs were taken by using Digital Instruments Multimode Scanning Probe Microscope (SPM) Nanoscope IV (Veeco Instrument Inc.). Images were taken in tapping mode using a silicon nitride (Si_3N_4) probe (length: 115-135 µm; width: 30-40 µm; frequency (f_0): 237-326 kHz, Spring constant (k): 20/80 N/M). Images were collected at 256 lines per scan with a scan rate of 0.2-0.5 Hz. Data were analysed using Nanoscope SPM V613b36.

Roughness of the conidia surfaces were compared from their Root Mean Square of the roughness value (RMS values) from the height images acquired by the AFM.

2.17 Insect rearing

2.17.1 *H. armigera*

The initial colony of *H. armigera* was established by collecting larvae and pupae of the insect from chickpea fields. Larvae were reared individually in sterile polypropylene vials (42-65 mm, 50 ml capacity; Laxbro Manufacturing Co., Pune, India) containing pieces of artificial diet (Nagarkatti and Prakash, 1974). They were maintained at 25-28ºC and 65-95% RH.

2.17.2 *S. litura* and *M. hirsutus*

Larvae of *S. litura* were reared in ethanol (90%) washed, plastic containers with a layer of filter paper at the bottom. They were maintained at 25-28˚C and 65-95% RH. First two larval instars were provided with tender castor (*Ricinus communis*) leaves surface sterilized with aqueous solution of sodium hypochlorite (0.5% v/v) followed by washing with sterile distilled water twice. From third instar onwards, the larvae were provided with surface sterilized mature leaves.

The mealy bugs of grapes (*M. hirsutus*) were reared on sprouted potato. They were maintained at 25-28˚C and 65-95% RH.

2.17.3 *Ae. aegypti*

Virus free adults of *Ae. aegypti* were obtained from Vector Control Research Centre (VCRC), Pondicherry, India and reared in cages under laboratory conditions of 28°C, 75% ± 5% RH and a photoperiod of 16:8 (L:D). Larvae were maintained in trays filled with water and fed with pet food and yeast powder (3:1).

2.18 Hemolymph collection

Hemolymph was collected from 3rd instar larvae of *H. armigera* infected with *M. anisopliae* after surface-sterilization with 95% ethanol. The larvae were bled by puncturing of the prolegs (*H. armigera*) and applying gentle pressure to extrude drops of hemolymph, which was collected individually from each larva into a chilled 1.5 ml tube. Hemolymph was immediately used for microscopy. The collected hemolymph was further used for enzyme activities.

2.19 Evaluation of conidial adhesion to the insect cuticle

Inoculation of insect and elimination of nonadhered conidia: 3rd instar larvae of *H. armigera* were washed with distilled water, allowed to air dry and then used for the adhesion experiments. The larvae were inoculated with *M. anisopliae* by dipping in conidial suspensions (2 ml of 1×10^7 conidia/ml per larvae) for 5 s. The inoculated larvae were air dried on filter paper at room temperature. After 0.5 h, the inoculated insects were washed with 0.1% Tween-80 (5 s, mixing) and then rinsed with tap water. These actions eliminated any conidia that had not really adhered to the cuticle but were only resting on the cuticle surface. After rinsing, the insect was left to dry on filter paper for 1 h.

Removing and counting adhered conidia: Each larva was placed in a polypropylene centrifuge tube (1 larva/tube) containing dichloromethane (DCM) and the tube was shaken for 5 min. The adhesion of the conidia to the tubes had been tested previously and found to be negligible. The solvent extract containing conidia was transferred to a new tube, mixed with ethyl alcohol (1:1) (99.8% analytical) and centrifuged for 30 min. at 6000 g. The supernatants were examined under a light microscope and the presence of conidia in these supernatants was found to be negligible. The conidia free supernatants were discarded and each of the conidia containing sediments was suspended in 1 ml of 0.1% Tween-80. The tubes were vigorously shaken and the conidial count was examined with a hemocytometer.

2.20 Bioassays

2. 20.1 Bioassay with *H. armigera*

Third instar larvae of *H. armigera* were used in bioassays with *M. anisopliae* conidia. The 7-day-old sporulating cultures of *M. anisopliae* isolates grown on PDA were used to prepare the conidia suspensions. Conidia were lightly scraped off the slant surface in 0.1% (w/v) Tween 80 to get a suspension with 1×10^7conidia/ml. Larvae were dipped individually in 10 ml conidia suspensions for 5 s. After treatment, each larva was individually transferred to a separate sterile vial containing moist Whatman No. 1 filter paper and a piece artificial diet. The diet was changed on alternate days and the larvae were kept at 25-28ºC, 65-95% RH and 16:8 (L: D) for 14 days. Dead larvae were transferred to sterile petri plates containing moist cotton

swabs and kept at 28ºC with 70-80% RH for at least 3-7 days to allow mycelial growth and conidia formation over the cadavers. The data of percent mortality from three experiments were pooled to get average values, which were corrected by Abbott's formula (Abbott, 1925). Studies to determine the median lethal time (LT_{50}) were carried out using *M. anisopliae* isolates M2104, M34311, M34412, M81123, M91427, M91629, M101133, M101335, M101537 and M16760. The estimates of LT_{50} were determined using probit analysis (Throne et al., 1995). A set of 30 larvae with three replications treated with 0.1% (w/v) Tween 80 in sterile distilled water served as control. The experiment was conducted 3 times using freshly prepared conidia suspensions.

Median lethal concentration (LC_{50}) studies for M34311, M34412, M81123, M91427 and M91629, were carried out with four concentrations of conidia: 1×10^3, 1×10^5, 1×10^7 and 1×10^9 conidia/ml to increase the possibility of identifying the difference in virulence of isolates with high mortality values that might go undetected if only a single dose was used. The experimental layout was a RCBD with each treatment containing a set of 30 larvae repeated 3 times. The estimates of LC_{50} were calculated manually using probit analysis according to Finney (1981).

2.20.2 Bioassays with *S. litura* and *M. hirsutus*

Third instar larvae of *S. litura* were used in bioassays of *Metarhizium* conidia. The fungal suspension was prepared as described above. After treatment, a set of 30 larvae were transferred to a separate plastic container containing filter paper and a castor leaves. The leaves were changed every other day and the larvae were kept at 25-28˚C, 65-95% RH and 16:8 (L: D) for 14 days. Dead larvae were transferred to sterile petri plates containing moist cotton swabs and kept at 28-28˚C at 70-80% RH for at least 3-7 days to allow mycelial growth and conidia formation over the cadavers.

The mealy bugs of grapes (*M. hirsutus*) were reared on sprouted potato and used for bioassay. Effect of entomopathogenic fungi (*M. anisopliae* 1×10^7 conidia/ml) on mealy bug of grapes was studied. Mealy bugs treated with phosphate buffer (pH 6.8, 50 mM) and distilled water containing 0.01% Tween 80 was also kept for comparisons. All the treatments were applied using an automiser (Hand-spraying device for laboratory bioassay). Each treatment containing the set of 20, 3rd instar

mealy bugs with 3 replications. After treatment, sprouted potatoes were kept in aseptic conditions containing moist filter paper and were incubated at 25±2 °C with 70-80% RH for 14 d. The mortality was recorded using a dissection microscope.

2. 20.3 Bioassays with *Ae. aegypti*

To evaluate fungal efficacy, i.e., the dose of conidia required, as well as the lethal exposure time and stability of the conidia, different bioassays were carried out against the larvae and adults of *Ae. aegypti*. Bioassays were also carried out to evaluate the efficacy of *M. anisopliae* M34412 conidia from 1st and 40th subculture and *M. anisopliae* M34412 (1st subculture) conidia in combination with *M. verrucaria* enzyme against larvae of *Ae. aegypti.*

2. 20.3.1 Larvae

Twenty 3rd instar larvae of *Ae. aegypti* were transferred to 50 ml glass beakers containing 20 ml of *M. anisopliae* conidial suspensions (1×10^3 to 1×10^7 conidia/ml) of 3 strains, *viz* M34412, M34311 and M81123 and 40th subculture of M34412 in tap water, separately. This was replicated 3 times to ensure reproducibility. The larvae in water without conidia served as control. Total survival was recorded for all the replicates of each treatment at 24 h intervals up to 7 d.

In another bioassay with larvae, varying concentration of *M. verrucaria* enzyme complex (measured in terms of total chitinase activity, 10-30 units), singly and in combination with *M. anisopliae* M34412 conidia at a concentration of 1×10^3 per ml were used. The observations were recorded for 7d at intervals of 24h.

2.20.3.2 Adults

M. anisopliae M34311, M34412 and M81123 strains were evaluated against adult females of *Ae. aegypti* by indirect contact method using filter paper impregnated with conidial suspensions. Sterile filter paper (Whatman No.1, 8×6 cm) was immersed in suspensions of 1×10^7 to 1×10^{10} conidia/ml. Corresponding conidia concentration on the paper were 1.39×10^6 to 5.9×10^8 conidia/cm^2 (estimated by determining conidial concentrations following re-suspension of conidia from randomly sampled 1 cm^2 of filter paper), respectively. The filter papers impregnated

with conidia (conidial cards) were allowed to dry at 28ºC, 70 % RH for 16-18 h, before being placed in 500 ml glass beakers. The size of the conidial card was such that it rested almost vertically within the beaker and allowed free movement of the adult mosquitoes in the beaker. Ten adult mosquitoes (2-3 d old) were released in each beaker and for each strain, the sample size was 30. Control treatments were carried out by treating the filter papers without conidia. All the experiments were conducted thrice and appropriate controls (filter papers treated with 0.1 % (w/v) Tween 80) were maintained. Insects were fed on 10 % sucrose in cotton swabs placed inside the beaker (Fig. 2.1). The observations were taken for 8 days.

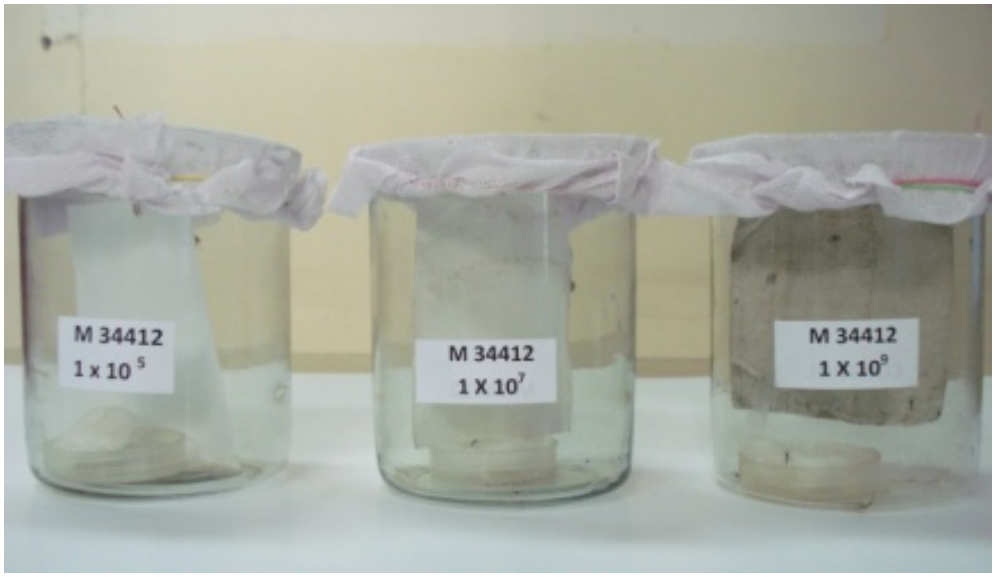


Fig. 2.1 Setup for bioassay of *Ae. aegypti* adult with *M. anisopliae* at different concentration

For determining the mean lethal exposure time, adult mosquitoes were exposed to filter papers impregnated with *M. anisopliae* M34412 (1×10^{10} conidia/ml) formulated in 0.1% (w/v) Tween-80 for different time periods *viz*., 1 h, 4 h, 12 h, 24 h and 48 h. The papers were removed after the indicated time and survival was recorded every 24 h for a period of 8 d.

The efficacy of conidia of *M. anisopliae* M34412 on impregnated filter paper was also studied. The efficacy of conidia was evaluated by exposing adult females of *Ae. aegypti* to the same treated filter papers after every 1 week upto 4 weeks.

The dead larvae and adults of *Ae. aegypti* treated with conidia of *M. anisopliae* were transferred to wet filter paper and maintained at 70-80 % RH and 28°C for

fungal growth. Conidial germination on cuticle was observed under an inverted light microscope. Hyphal growth, indicative of fungal infection, was observed after 3-4 d.

2.21 Field experiment

Performance of the three *M. anisopliae* isolates was evaluated by applying conidia against *H. armigera* in chickpea (variety Vishal) fields with the chemical insecticide endosulfan and untreated plots were maintained as controls. Field experiments were conducted at the Agriculture University, Rahuri, Ahmednagar, Maharashtra (19º20'N and 74º35'E) for two successive years during *Rabi* season (October-March) 2005-2006 and 2006-2007. The experimental layout was a RCBD with five treatments and three replications. The plot size was 5×5 m. The crop was sown during the first fortnight of October and was raised following normal agronomical practices.

Based on the previous criteria, the three selected *Metarhizium* isolates M34311, M34412 and M81123 with dosage of 5×10^{12}conidia/ha in 0.1% (w/v) Tween 80 suspension and endosulfan at 350 g.a.i./ha were sprayed with a hand operated knapsack sprayer. Control plots were sprayed with 0.1% (w/v) Tween 80. As per agricultural practices for the chickpea crop, insecticides were first sprayed 10-15 days after egg lying and thereafter 2 more times with a 14-day interval. Spraying was carried out between 16:00 and 18:00 h. Precautions such as monitoring wind direction and using cloth curtains as necessary were taken to avoid wind drift to neighbouring plots.

Five plants per plot were randomly selected and tagged for recording observations. Live larvae were counted before treatment and after each treatment on the 3^{rd}, 7^{th} and 10^{th} days. The percent efficacy for each treatment was calculated according to Henderson and Tilton (1955). The observations of pod damage were recorded on five randomly selected plants at the time of harvest by counting the total number of healthy and damaged pods. The percent pod damage was calculated and the percentages were further transformed to arcsine values for statistical analysis. The data on larval population for the 2 years of field evaluations was converted to square root n+0.5 transformations. The data were then subjected to analysis of variance and the means were compared using F-test at $\alpha=0.05$ level of a critical difference.

2.22 Statistical analysis

F-test- The percent viability was transformed to arcsine values to improve the homogeneity of variances. The data were then subjected to analysis of variance and the means were compared using F-test at α-0.05 level of critical difference as described by Panse and Sukhatme (1989).

Probit analysis- The estimates of LT_{50} and LC_{50} were calculated manually using probit analysis according to Finney (1981). For both LT_{50} and LC_{50}, observed chi-square values were compared with tabulated Chi-square values.

Chapter 3

Screening of *Metarhizium* isolates for the control of *Helicoverpa armigera*

M. anisopliae is one of the promising mycoinsecticides because of its high virulence, specificity and with no adverse effect on the environment (Ignacimuthu, 2008). *M. anisopliae* has been employed against a variety of insect pests including *H. armigera* (Rijal et al., 2008). The pod borer, *H. armigera* (Lepidoptera: Noctuidae), is a worldwide pest of important horticultural and agricultural crops (Fitt, 1989), including chickpea (Mehto et al., 1985; Pawar, 1998; Pande et al., 2000), pigeon pea, mungbean (Shanower et al., 1999) and cotton (Sun et al., 2002).

Development of an effective mycoinsecticide depends on selection of a strain that is stable and virulent against the target host (Milner et al., 2002). They cause infection by growing through the body of insect and release extracellular cuticle degrading enzymes (chitinase, chitosanase, chitindeacetylase, protease and lipase). Moreover, these fungi release toxins which are one of the important killing components in entomopathogenesis. Thus, various fungal strains differ in their host specificity, virulence factors such as toxins and CDE complex production (Petlamul and Prasertsan, 2012).

In the present study, sixty three *Metarhizium* isolates obtained from soils and insect cadavers from different fields were screened for the control of *H. armigera*.

Results and discussion

3.1 Isolation of *Metarhizium* isolates

Using selective media (Table 2.2), a number of isolates belonging to different entomopathogenic genera were obtained from the soil and insect samples. The isolates were identified on the basis of conidial morphology (Barnett and Barry, 1999) and 63 *Metarhizium* (dark green conidia) isolates obtained were used in the present study.

From the 63 *Metarhizium* isolates, 53 were obtained from soil samples whereas 10 were from insect cadavers (Table 3.1). Sahayaraj and Borgio (2009) isolated effective *M. anisopliae* from soil samples collected from different crop fields of Tirunelveli district of Tamil Nadu (October 2005). Bidochka et al. (2001) reported that *M. anisopliae* could survive in soil in the absence of an insect host and that the habitat could influence the evolution of fungus-host interactions. Presumably, *Metarhizium* species can survive very well and grow saprophytically in the soil, suggesting that all the sixty three isolates from the present study may not have an effective bio-control potential.

Table 3.1 Origin of *Metarhizium* isolates

Source: Soil (53 isolates)	**Field**	**Total isolates**
M1311, M1322, M1333, M2104, M2305, M2416, M2427, M2508, M42014, M45115, M45216, M45317, M79120, M79221, M79322	Tomato	15
M3419, M34210, M34311, M34412, M34513	Custard apple	5
M81123, M91124, M91225, M91326, M91528, M91427, M91629, M91730, M91831, M91932, M111145	Sugarcane	11
M101133, M101234, M101335, M101436, M101537, M101638, M101739, M101840, M101941, M102042, M102143, M102244	Brinjal	12
M51118, M51219	Okra	2
M131150, M141151, M141252, M151153	Pigeon pea	4
M121146, M121247, M121348, M121449	Chickpea	4
Source: Insect hosts (10 isolates)	**Insect**	**Total isolates**
M16255, M16356, M16457, M16558, M16659	Pigeon pea-Greasy cutworm	5
M16154, M16760	Sugarcane-Mealybug	2
M16861	Sugarcane-White grub	1
M16962	Sugarcane-Beetle	1
M161063	Sugarcane-*Pyrilla perpussila*	1

Identity number (I.D.) for soil isolates was based on crop, geographical location, plot, sub-plot if any, sample and isolate number.

3.2 Evaluation and selection of *Metarhizium* isolates based on mortality against *H. armigera*

The sixty three *Metarhizium* isolates were tested for their biocontrol efficiency against 3rd instar larvae of *H. armigera*. All the sixty three isolates of *Metarhizium*

were pathogenic to *H. armigera*. Higher percent mortality was observed after 3-4 days treatment for all the isolates. However, the percent mortality varied from 28-97% for different *Metrhizium* isolates. Only 10 isolates showed >90% mortality, whereas, 18 isolates showed mortality between 80-90% mortality and rest of the isolates exhibited mortality between 25-80%. (Table 3.2). Surprisingly, only one isolate obtained from insect cadaver showed mortality above 90%. The most virulent isolate M34412 obtained from soil sample of a custard apple field showed 97.33% mortality.

Many studies from Indian subcontinent have demonstrated the potential of *Metarhizium* for the control of *H. armigera* (Kencharaddi and Jayaramaiah, 1997; Rijal et al., 2008; Sahayaraj and Francis 2010; Vijayavani et al., 2010). For determining percent mortality, the larvae were dipped in 1×10^7 conidia/ml solution of *Metarhizium* isolates. Vijayavani et al. (2010) had reported two strains of *M. anisopliae* isolated from Hyderabad (Nandigama village and Medchal) with >90% mortality of *H. armigera* larvae at 1×10^7 conida/ml concentration. Four *M. anisopliae* strains isolated from insects and soils from different parts of Nepal were found to be pathogenic to 3rd instar larvae of *H. armigera* at 1×10^7 conidia/ml concentration, but with great variability among the isolates (Rijal et al., 2008). Sahayaraj and Francis (2010) observed that 93.33% of mortality against 3rd and 4th instars of *H. armigera* at 4 d after the treatment of *M. anisopliae*. Gundannavar et al. (2007) reported that *M. anisopliae* was more virulent to the first instar larvae of *H. armigera*. At higher concentrations of 10^8 and 10^7conidia/ml, 100% mortality was observed, while

Fig. 3.1 *M. anisopliae* pathogenicity towards 3rd instar larvae of *H. armigera*
1) Healthy larvae, 2) Dead larvae (4 d after conidia application), 3) Dead larvae with thick fungal mat (5 d after conidia application), 4) Dead larvae covered with conidia (6 d after conidia application)

concentration of 10^5, 10^3 and 10^2 conidia/ml resulted in 72.5, 52.5 and 27.5% mortality. *M. anisopliae* also has the potential to cause high (90% or greater) mortality to *Agriotes obscurus* (Kabaluk et al., 2001).

After death of the larvae, stiffness of the cadaver, formation of mycelial mat and sporulation covering the cadaver was observed on 4^{th}, 5^{th} and 6^{th} d respectively (Fig 3.1). Zimmermann (1982) reported that *M. anisopliae* required 5 days for mycosis under optimal temperature and humidity.

Based on percent mortality, 10 isolates showing >90% mortality (Table 3.2) were selected for the determination of LT_{50}.

Table 3.2. Evaluation of *Metarhizium* isolates on the basis of mortality against *H. armigera*

S. No.	Isolates	Mortality (%)
1	M34412, M34311, M81123, M91629, M101335, M2104, M101133, M91427, M16760, M101537	>90
2	M34210, M1333, M34513, M45317, M34216, M102143, M101436, M141252, M102244, M2427, M16154, M16558, M91124, M161063, M101638, M1322, M16962, M2416	80-90
3	M121449, M16356, M121146, M111145, M16255, M91326, M101234, M91326, M101234, M2305, M1311, M141151	70-80
4	M102042, M101739, M16659, M101941, M16861, M151153, M16457, M121247, M42014, M79322, M79120	60-70
5	M3419, M2508, M91225, M101840, M45115, M91831, M121348	45-60
6	M131150, M91932, M51219, M91730, M79221	25-45

3.3 Determination of Median Lethal Time (LT_{50})

Day-wise mortality was recorded to determine the fastest kill time required for the ten *Metarhizium* isolates. *Metarhizium* isolate M34412 recorded lowest LT_{50} (3.3 days), whereas, LT_{50} values were 3.3, 3.5, 3.6 and 4.1 days for M81123, M34311, M91629 and M91427, respectively (Table 3.3). The remaining isolates had relatively

Table 3.3 Median Lethal Time (LT_{50}) of 10 selected isolates of *Metarhizium* against 3rd instar larvae of *H. armigera*

Isolate No.	Chi-square value	LT_{50} (d)	Fiducial limit (d)
M34412	2.74 (14.07)	3.3	3.0-3.6
M81123	1.96 (15.51)	3.3	3.1-3.6
M34311	0.67 (18.31)	3. 5	3.2-3.7
M91629	4.05 (15.51)	3.6	3.3-3.9
M91427	2.28 (15.51)	4.1	3.7-4.4
M2104	10.79 (12.59)	4.8	4.5-5.1
M101335	0.69 (15.51)	4.6	4.2-5.0
M101537	10.16 (15.51)	6.4	6.1-6.7
M101133	2.03 (15.51)	6.7	6.3-7.0
M16760	2.50 (15.51)	6.8	6.4-7.1

higher LT_{50} values with M16760 showing the highest LT_{50} of 6.8 days. All Chi-square values were not significant ($\alpha = 0.05$) indicating good fit of regression lines. Within the 10 isolates, least percent mortality and highest LT_{50} value were observed for *Metarhizium* M16760, the isolate from the mealybug, which may be due to host specificity of that particular *Metarhizium* isolate. The LT_{50} values against *H. armigera* for seven isolates (3.3-4.6 d) from present study were better than four *M. anisopliae* isolates (ranging LT_{50} 5.3-6.8 d) previously reported by Rijal et al. (2008).

Based on the LT_{50} results, M34311, M34412, M81123, M91427 and M91629 were selected for Median lethal time (LC_{50}) experiment.

3.4 Determination of median lethal concentration (LC_{50})

The selected five isolates, at 1×10^3, 1×10^5, 1×10^7, 1×10^9 conidia/ml concentrations were used in the bioassays to determine the median lethal concentration (LC_{50}) i.e. number of conidia required to kill 50% third instar larvae of *H. armigera.* Isolate M34412 was more virulent than other isolates in this experiment based on LC_{50} values. *Metarhizium* M34311 and M81123 displayed low LC_{50} values ($<6\times10^3$ conidia/ml) while M91427 and M91629 showed high LC_{50} values ($>16\times10^3$ conidia/ml). All Chi-square values were not significant ($\alpha = 0.05$) indicating a good fit of regression lines (Table 3.4). Nguyen et al. (2007) reported LC_{50} 6.0×10^5 conidia/ml

Table 3.4 Median Lethal Concentration (LC_{50}) of 5 selected isolates of *Metarhizium* against 3rd instar larvae of *H. armigera*

Isolate No.	Chi-square value	Slope	SE of slope	LC_{50} ($\times 10^3$ conidia/ml)	Fiducial limit ($\times 10^3$ conidia/ml)
M34412	2.50 (5.99)	0.246	0.0762	1.40	0.1-1.9
M34311	1.47 (5.99)	0.299	0.0772	2.04	0.4-10.3
M81123	2.95 (5.99)	0.281	0.0727	5.7	1.2-26.7
M91629	3.31 (5.99)	0.266	0.0666	16.80	4.5-62.3
M91427	0.096 (5.99	0.299	0.0726	35.82	9.1-140.7

of *M. anisopliae* against 3rd instar *H. armigera,* whereas, Sahayaraj and Francis (2010) observed *M. anisopliae* LC_{50} of 1.25×10^6, 1.75×10^6 and 2.40×10^5 for 2nd, 3rd and 4th instar larvae of *H. armigera,* respectively. Kumar and Chowdhury (2004) reported *M. anisopliae* LC_{50} of 1.23×10^3 conidia/ml against 2nd instar of *H. armigera*. The concentration of conidia required to kill 50% third instar larvae of *H. armigera* was at least 10 fold less for all the isolates as compared to above mentioned reports. The results of percent mortality, LT_{50} and LC_{50} values indicated that *Metarhizium* M34412, M34311 and M81123 isolates were best among all and were further studied for production of conidia on a solid substrate, viability, settling time, CDE production and field application.

3.5 Production, viability and settling time for conidia of *Metarhizium* isolates

The three *Metarhizium* isolates M34412, M34311 and M81123 were identified up to species level as strains of *M. anisopliae* by ITS sequencing (Kulkarni et al., 2008). Conidia production was carried out with the three selected strains using rice as a substrate. The conidial yields were not significantly different from each other. The conidial yields for all three strains were in the range of 60-75 g/kg substrate and the numbers of conidia/g were in the range of 4.04×10^{10}- 4.4×10^{10}. Vijayavani et al. (2010) inferred that virulent isolates of *M. anisopliae* are necessarily fast in germination thereby suggesting it to be an important criterion for identifying virulent isolates.

In present study, the percent conidia germination on PDA agar ranged from 92 to 97% after 24 h for all the three strains (Table 3.5). Whereas, Petlamul and

Table 3.5 Conidia production, sedimentation time and % germination of the selected strains of *M. anisopliae*

Isolate	Yield (g/kg rice) mean±SD	ST_{50} in Tween-80 (h)	Fiducial limit (h)	Germination (%) mean±SD
M34311	60±2.64	2.47	2.26-2.69	92±2.64
M34412	67±3.46	2.30	2.11-2.52	97±1.73
M81123	75±3.60	2.65	2.43-2.90	93±1.73

Prasertsan (2012) observed that *M. anisopliae* conidia germination on Czapeck Dox agar ranged between 70-72% after 48 h. The hydrophobicity of conidia is one of the essential characteristics for adhesion on the insect cuticle and higher the hydrophobicity of the conidia, faster is the sedimentation. The ST_{50} values determined using 0.2 M $(NH_4)_2SO_4$ were 2.1, 2.2 and 2.5 h for *M. anisopliae* M34412, M34311 and M81123, respectively. For the bioassays as well as for spraying in the field, 0.1% (w/v) Tween 80 was used to maintain homogenous conidial suspensions. Therefore, the sedimentation of conidia in 0.1% (w/v) Tween 80 was also studied. It can be seen from Table 3.5, the ST_{50} were in the range of 2.30 - 2.65 h in Tween 80. *M. anisopliae* M34412 conidia settled faster indicating greater hydrophobicity than the other two strains.

3.6 Production of cuticle degrading enzymes

Penetration of the insect cuticle by entomopathogenic fungi requires the production of cuticle degrading enzymes so as to degrade the highly refractory insect cuticle. The levels of different enzymes, essential for cuticle degradation, produced by the 3 isolates in the chitin medium are given in Table 3.6. Cuticular chitin is shielded

Table 3.6 Extracellular cuticle degrading enzyme activities produced by *M. anisopliae* strains

Isolate No.	Chitinase ($\times10^{-3}$ U/ml)	CDA ($\times10^{-3}$ U/ml)	Chitosanase ($\times10^{-3}$ U/ml)	Protease (U/ml)	Lipase (U/ml
M34311	3.53±0.19	3.21±0.22	35.7±2.10	3.28±0.21	0.74±0.08
M34412	3.95±0.11	1.30±0.19	32.39±1.12	3.38±0.15	0.99±0.04
M81123	3.76±0.24	2.47±0.21	35.8±1.63	3.32±0.21	0.74±0.04

by proteins and lipids, which needs to be removed by proteases and lipase for subsequent action of chitinolytic enzymes. Higher mortality, LC_{50}, LT_{50} observed for *M. anisopliae* M34412 may be partly attributed to the high levels of lipase and protease produced by it. St. Leger et al. (1986) reported that the presence of an insect cuticle or chitin in the medium induced sequential production of protease, lipase and chitinase in response to the cuticular composition.

Petlamul and Prasertsan (2012) observed that the chitinase activity of *M. anisopliae* appeared after 72 h and increased up to 96 h and maintained constant upto 120 h. Ramanujam et al. (2011) reported that thirty three isolates of *M. anisopliae* exhibited chitinase activity ranging from 23 to 144 µg/ml. Constitutive production of CDA and chitosanase in YPG medium have also been reported to be important in fungus-insect interactions (Nahar et al., 2004).

3.7 Field performance studies

Field performance of the three *M. anisopliae* strains, M34311, M81123 and M34412 was evaluated at the Agriculture University, Rahuri, Ahmednagar, Maharashtra (19º20'N and 74º35'E) for two successive years during the *Rabi* season (October-March) of 2005-2006 and 2006-2007. Control of *H. armigera* in chickpea fields using conidia of *M. anisopliae* strains was compared with the efficacy of chemical insecticide endosulfan, which is banned since April 2011 in india and the untreated (sprayed with 0.1% (w/v) Tween 80) plots. Differences in the larval population due to different treatments were recorded up to 42 days (Fig. 3.2). Larval survival was highest in the control. The mean numbers of surviving larvae per plant were 1.6, 1.6, 2.1 and 2.1 after treatment with endosulfan, M34412, M34311 and M81123, respectively, and 5.4 in the untreated control (Fig. 3.2). The mean percent efficacies with endosulfan and M81123, M34311 and M34412 were in the range of 65-74%. The percent pod damage in the untreated control was 42% while in the treated plots it was between 17 and 26%. Correspondingly, the grain yields were also higher. The percent efficacy obtained with endosulfan was 74%, while treatment with the *Metarhizium* isolates gave 65 to 72% efficacy (Table 3.7).

Large-scale field trials and commercial trials reported by other groups showed that after the fungus provided 50- 60% control of the pest in the season of application

Table 3.7 The field performance studies of *M. anisopliae* strains to control *H. armigera* in chickpea field

Treatments	Efficacy (%) mean±SD	Pod damage (%) mean±SD	Grain yield (kg/ha) mean±SD
Endosulfan	74.0±5.9a	17.5±10.6a	2053±2.2c
M34412	72.2±20.5a	19.5±10.6a	1878±4.2b
M34311	65.2±21.1a	26.3±10.6b	1610±6.4b
M81123	65.8±19.9a	21.4±10.6a	1662±3.2b
Untreated control	-	42.1±10.6c	1013±3.6a

The percent efficacy was calculated using method described by Henderson and Tilton (1955) over untreated control. Numbers followed by the same letters within a column are not statistically different. SD, standard deviation.

and persisted to provide effective control in ratoon (sugarcane) crops (Robertson et al., 1996; Logan et al., 1999).

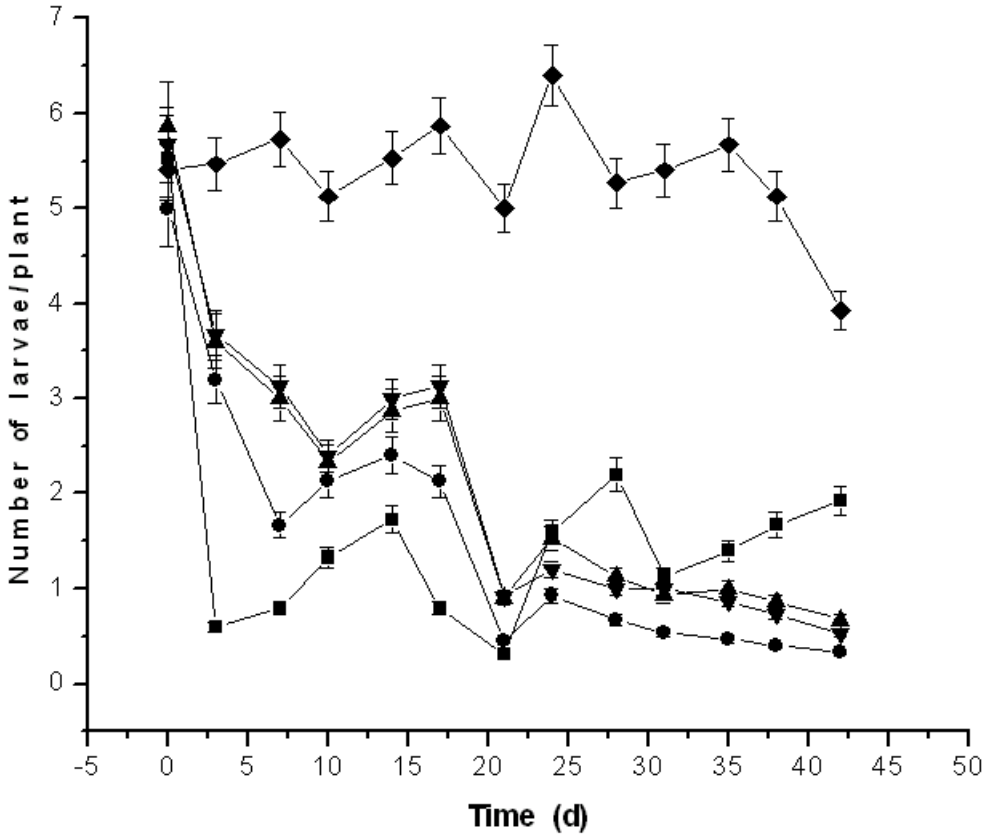


Fig. 3.2 The effect of *M. anisopliae* strains and endosulfan on the larval population of *H. armigera* on chickpea under field conditions.(■—■), Endosulfan; *M. anisopliae* strains (▼-▼) M34311; (●-●), M34412; (▲-▲), M81123; and (▼—▼), untreated control.

In conclusion, all three *M. anisopliae* strains, M34412, M34311 and M81123 were found to be virulent to the target pest under field conditions and possess traits necessary for development of successful mycoinsecticide.

Chapter 4

Metarhizium anisopliae pathogenesis in *Helicoverpa armigera* and factors involved in their interaction

A. Pathogenesis of *M. anisopliae* in *H. armigera*

Infection of an insect by entomopathogenic fungus occurs by a series of events. The process can be divided into three parts: adhesion of the fungal conidia, penetration through the cuticle and establishment within the host. Askary et al. (1999) reported that conidial adhesions to host surface and integument penetration were the initial events during the pathogenic process. According to Boucias et al. (1988) and Boucias and Pendland (1991) conidial attachment to the cuticle involves nonspecific adhesion mechanisms mediated by the hydrophobicity of the conidial cell wall. Most of the work examining adherence and penetration of conidia was carried out using *M. anisopliae* (Metschnikoff) (St. Leger et al., 1993; Hajek and St. Leger, 1994). Growth of the fungi in the hemocoel may be as yeast like blastospores, hyphal bodies or protoplasts rather than mycelium. The switch in the morphological form allows dispersion and colonization of the hemocoel, optimized nutrient acquisition by increasing surface area and dispelling of the efforts of the host cellular immune system. Such proliferation within the tissues and the haemolymph leads to death of the host and eventual eruption through the host cadaver.

The pathogenesis of *M. anisopliae* in *H. armigera* as evidenced through scanning electron and light microscopy of the different phases of fungal infection is presented in following sections.

Results and discussion

4.1 Adhesion

Scanning electron microscopic analysis of infected *H. armigera* revealed that conidia of *M. anisopliae* are capable of attachment anywhere on the epicuticle surface, although preferred attachment sites were insect setae, hair socket joints, legs and head (Fig. 4.1 a, b). Other studies with *M. anisopliae* also documented that the cuticle regions containing a large number of setae served as high-affinity sites for conidial adhesion (Goettel et al., 1989; Boucias et al., 1988). Whereas, Vestergaard et al. (1999) observed that *M. anisopliae* conidia were capable to bind to any site on the cuticle of adult *Frankliniella occidentalis*, but were frequently trapped by the setae on

the wings and legs. However, McCauley et al. (1968) reported that infection sites varied with host species and most infections were found to occur in the membranous inter-segmental regions. Following application of *M. anisopliae* M34412 conidia, they adhere and consolidate on the insect integument within 6 h (Fig 4.1 c).

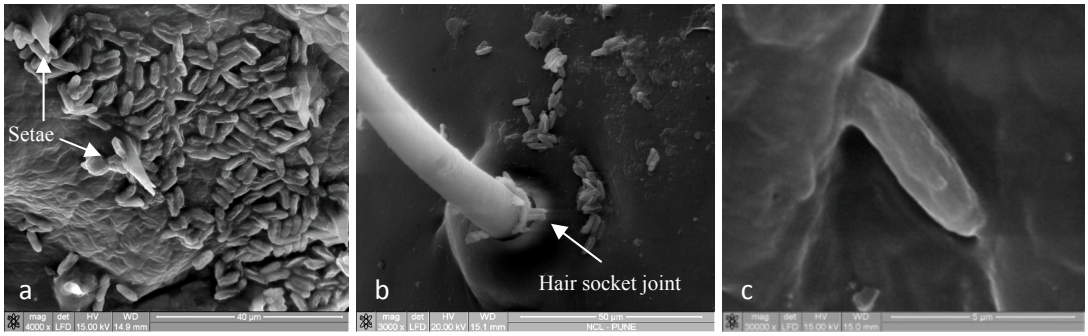


Fig. 4.1 Scanning electron microscopy images of conidia adhered to the epicuticle **(a and b)** (5000x) and consolidation of conidium **(c)** (30000x)

4.2 Conidia germination and appressorium formation

Conidial germination started after 12 h of inoculation (Fig. 4.2 a). Each conidium usually produces only one germ-tube, there being a variation in its length prior to appressoria formation; some conidia produce long germ-tubes whereas others produce shorter ones. The first sign of conidial germination is the extrusion of a germ tube. Gunnarsson (1988) reported that *M. anisopliae* conidia germinate within 12 h post-infection and epicuticle penetration occurs 12-18 h post-infection in *Schistocerca gregaria*. In *Manduca sexta*, the conidia germinate to form appressoria and penetrate the cuticle surface within 40 h post-infection (St. Leger et al., 1996b). Vestergaard et al. (1999) studied *M. anisopliae* infection of *Frankliniella occidentalis* and reported that conidia germinated within 24 h post-infection. Lecuona et al. (1991) observed that the conidia of *B. bassiana* germinate within 18 h after inoculation on *Ostrinia nubilalis* larvae. Whereas, according to Boucias et al. (1996), *B. bassiana* form germ-tubes and penetration structures between 12 and 24 h after inoculation in *Reticulitermes flavipes*. Similar variations have been observed in the case of *N. rileyi* where the conidial germination period varies from 8 to 48 h on different insect larvae (Kish and Allen, 1976; Mohamed et al., 1978; Boucias and Pendland, 1982; Thorvilson et al., 1985; Kumar and Dahiya., 1997). Conidial germination takes

relatively longer (24-48h) in Coleoptera hosts as reported in *Elateridae* grubs (McCauley et al., 1968). Conidial germination and penetration rates on the insect integument have been shown to be related to the virulence of the fungi and susceptibility of the insect host (Pekrul and Grula, 1979).

The globular appressoria of *M. anisopliae* were formed after 24 h on the larval cuticle of *H. armigera*. The appressoria were at the end of the germ-tubes with most of them being covered by a thin amorphous mucilage layer that firmly adhere the appressoria to the insect integument (Fig. 4.2 b). Similarly, *B. bassiana* has been shown to form appressoria on cuticles of other host insects, such as *Ostrinia nubilalis*, *Melolontha melolontha* (Lecuona et al., 1991) and *Leptinotarsa decemlineata* (Say) (Vey and Fargues, 1977).

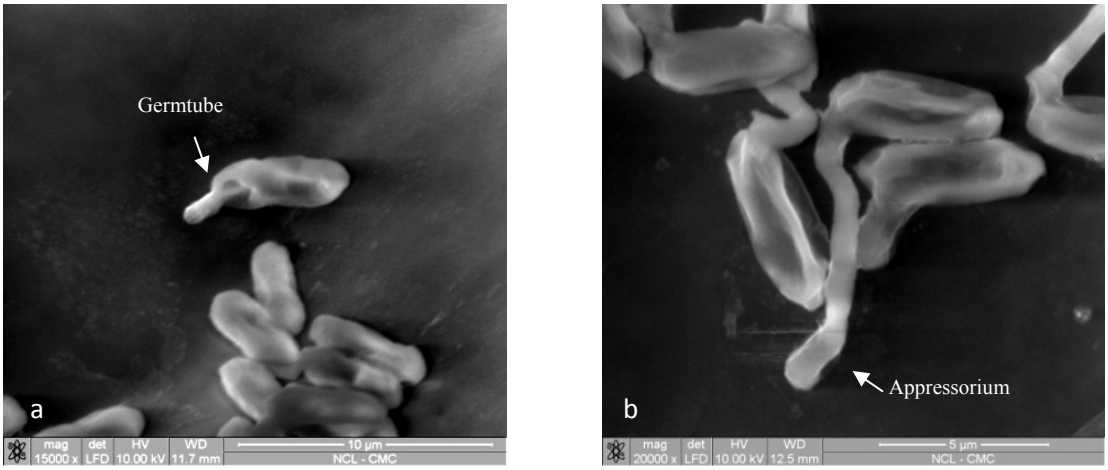


Fig. 4.2 Scanning electron microscopy images of germinating conidia **(a)** (15000x) and appressorium formation at the end of germtube **(b)** (20000x)

4.3 Penetration, colonization and mycosis

The penetration of entomopathogenic fungi is similar to that of plant pathogenic fungi and it is suggested to be based upon a combination of mechanical pressure and enzymatic degradation (St. Leger et al., 1987b). Direct penetration of the insect cuticle by germ tubes was also observed after 24 h, without any detectable appressoria differentiation (Fig. 4.3 a). Pekrul and Grula (1979) also showed direct penetration of the cuticle of corn earworm, *H. zea* by *B. bassiana* without appressoria formation.

The extracellular mucilage has been proposed to play a role in the support and transport of cuticle-degrading enzymes (St. Leger et al., 1996a). Potentially, the primary function of many of the enzymes associated with conidia, including those of *M. anisopliae*, is to hydrolyze the epicuticular wax layer and provide nutrients required for germ tube formation. The disappearance of the wax layer beneath appressoria of *M. anisopliae* on the wireworm cuticle indicates enzymatic activity (Zacharuk, 1981). *M. anisopliae* produces a variety of hydrolytic enzymes such as proteases, chitinases and lipases on the host cuticle during the infection process (St. Leger et al., 1987b, 1996a; Tiago et al., 2002; De Moraes et al., 2001).

In the present study, penetration was observed between 24 to 48 h after infection. The average time for the penetration of *M. anisopliae* in *Nasutitermes exitiosus* was reported to be 48 h (Hanel, 1982). Presence of clear zone or a halo surrounding the germ tubes after 36 h may be due to enzymatic degradation (Fig. 4.3 b). Similar 'circular haloes' were observed by Pekrul and Grula (1979) around the

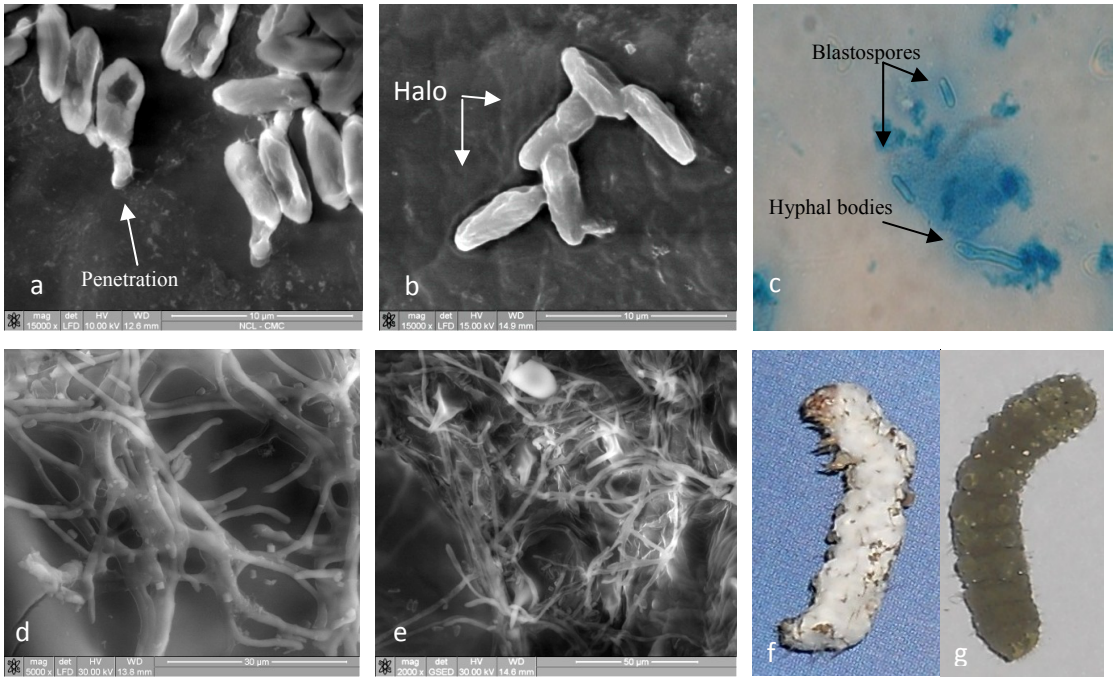


Fig. 4.3 Scanning electron and light microscopy images of direct penetration of the germ tube inside the insect body **(a)** (15000x) conidial penetration with halo formation in the cuticle (**b)** (15000x) hyphal bodies and blastospores from infected insect hemocoel **(c)**, extrusion of the mycelium **(d and e)** (5000x and 2000x) *M. anisopliae* mycelial layer on the insect cadaver **(f)**, *M. anisopliae* conidiogenesis on insect cadaver **(g).**

germ tubes of *B. bassiana* at the point of entry in *H. zea* larvae.

Around 48 h post infection, hyphal bodies and blastospores were observed in the haemolymph (Fig. 4.3 c). Extensive tissue invasion and degradation over the next 24 h led to the death of the host within 72-96 h. Fang et al. (2009) reported that after penetration, the hypha in the haemolymph differentiated to blastospores. These cells facilitated the spreading of the fungus to all organs thereby causing a generalized infection. In ticks however, Ment et al., (2012) reported that hyphal bodies of *Metarhizium* sp. did not commonly colonize and circulate within the haemolymph as long as the tick host was alive. Thus, death in ticks was due to cuticle penetration rather than by haemolymph invasion.

After death of the host larvae, hyphae re-emerged from the insect cadaver between 72 and 120 h. Though not visible to naked eyes, the extrusion and growth of the mycelium over thoracic region after 72 h can be visualised by SEM (Fig. 4.4). Mycelial extrusion from the cadavers between 72 and 96 h was mainly in the intersegmental areas and later, in areas with stronger cuticle, inducing complete cuticle degradation (Fig. 4.3 d and e). Subsequently, around 120 h, the whole body of the larvae was covered with a white mycelial layer (Fig. 4.3 f). Conidiogenesis was

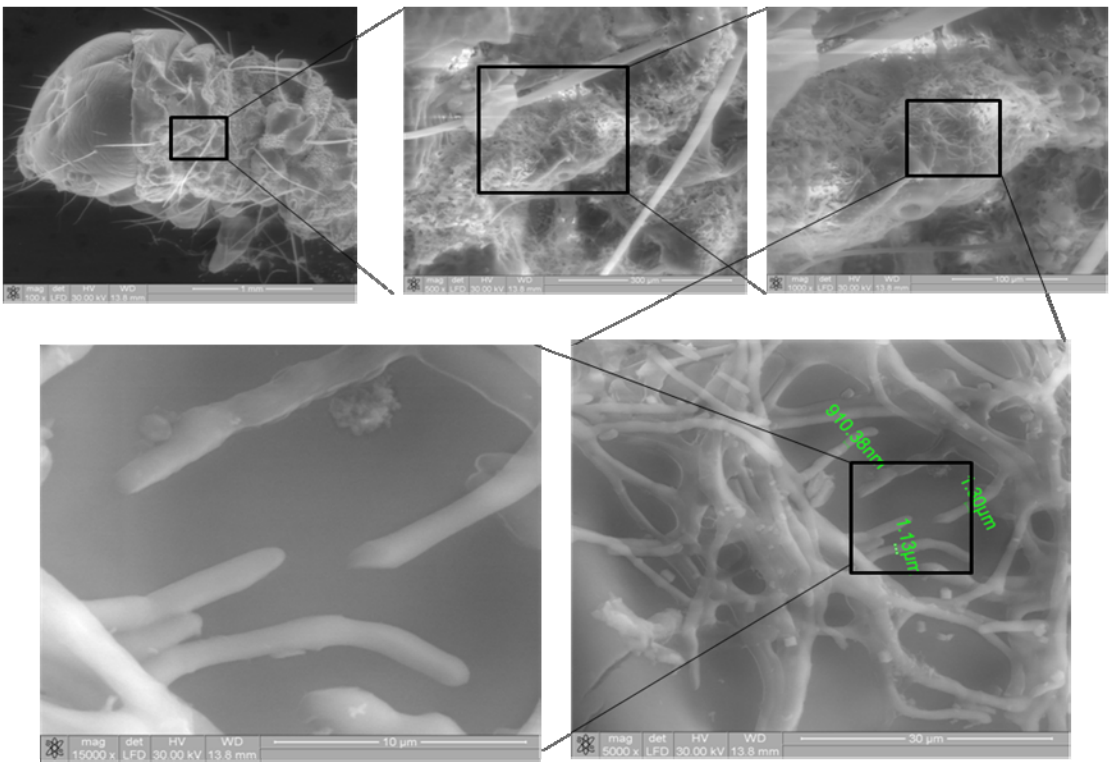


Fig. 4.4 Extensive growth of mycelium near insect thoracic region

observed on the insect cadaver between 120 and 144 h after inoculation (Fig. 4.3 g). The timeline for the different phases of the cycle of *M. anisopliae* pathogenesis in *H. armigera* is shown in Fig. 4.5. Small and Bidochka (2005) observed that as the host colonization by *M. anisopliae* proceeds, nutrients become exhausted and the fungus produces hyphae that emerge and produce conidia on the surface of the dead host of *Galleria mellonella*. In *Cornitermes cumulans*, greater amounts of mycelial extrusion points and conidiogenesis were observed in both insect legs and head region (Neves and Alves, 2000).

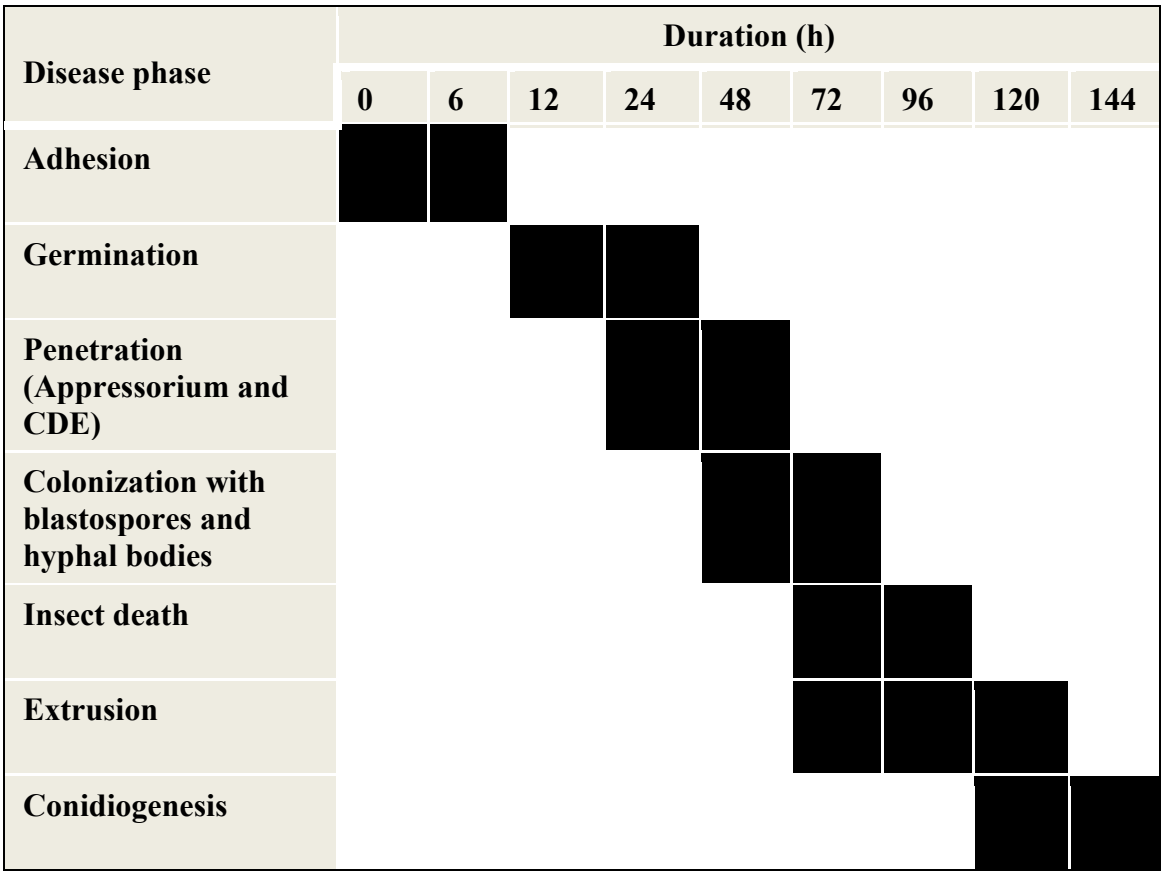


Fig. 4.5 Duration (h) of the different developmental phases of *M. anisopliae* on *H. armigera*

B. Effect of repeated subculturing on surface properties of *M. anisopliae*

One of the key attributes of a virulent strain is good adhesion of conidia to the surface of host cuticle (Shah et al., 2007). Adherence of more number of conidia is one of the factors leading to faster killing of the host; hence, poor adhesion is

considered as a feature of less virulent strains (Altre et al., 1999; Inglis et al., 2002). The cuticle of the host represents the first barrier to the pathogen and attachment of fungal propagules to the cuticle is the initial event in establishing mycosis. During adhesion, non-specific hydrophobic interactions were found to be involved between the conidial surface and the insect epicuticle (Boucias et al., 1988; Boucias and Pendland, 1991). Thus, the surface properties of the fungal conidia form the basis for the host-pathogen interaction. The surface properties are dependent on the factors that contribute to the adhesion process, including electrostatic charge and hydrophobicity. Many other factors may affect the adhesion of conidia to the host cuticle i.e., characteristics of the pathogen, including its virulence, conditions under which the pathogen is cultured, type of conidia, topographical and chemical properties of the host cuticle, host surface hydrophobicity, host behaviour and environmental conditions (Altre et al., 1999; Yaginuma et al., 2004; Ibrahim et al., 2002; Boucias et al., 1988; Butt, 1995; Sosa-Gomez, 1997; Hajek and Eastburn, 2003; Lacey et al., 1988; Quintela et al., 1998). The surface properties of conidia can be studied by determining their adhesion to polystyrene, roughness, charge and hydrophobicity (Amaral et al., 2006; Holder et al., 2007; Shah et al. 2007).

Entomopathogenic fungi like *M. anisopliae, B. bassiana, Nomuraea rileyi, Paecilomyces farinosus* and *Verticillium lecanii* loose their virulence over continuous subculturing on artificial media, which may be regained by passage through their respective insect hosts (Boucias et al., 1988; Brownbridge et al., 2001; Nahar et al., 2008). Previous report from our laboratory has showed that repeated *in vitro* sub-culturing of *M. anisopliae* M34412 resulted in decline in virulence which may be attributed to the decline in hydrolytic enzyme activities (Nahar et al., 2008). Change in the surface properties of the conidia may be another important contributor for the decreased virulence. Therefore, it was decided to study the surface characteristics of 1st and 40th subculture conidia of *M. anisopliae* M34412 and possible role of hydrophobins in fungus-insect interaction.

Results and Discussion

Effect of repeated subculturing of *M. anisopliae* on conidial germination, appressorium formation and production of cuticle degrading enzymes has been tested

and was found to be same as reported earlier (Nahar et al., 2008). Therefore surface properties of 1st and 40th subculture conidia were studied to evaluate their role in fungus-insect interactions.

4.4 Characterization of surface properties of 1st and 40th subculture conidia

4.4.1 Settling time

The *M. anisopliae* conidia from 1st subculture settled faster (ST_{50} 2.30 h) as compared to that of the 40th subculture (ST_{50} 2.70 h) (Table 4.1). The settling time, a desirable property of any strain, is found to be affected by cell wall architecture, chemical composition and surface properties.

The roughness of the cell surface, which is due to the hydrophobicity, was found to affect the rate of sedimentation (Joshi et al., 2010). Higher the roughness, faster is the sedimentation. This indicated that the surface of conidia from 1st sub-culture may be more rough and hydrophobic than 40th sub-culture. Shah et al. (2007) also reported decrease in the hydrophobicity after repeated subculturing.

4.4.2 Adhesion to polystyrene

The adhesion to polystyrene assay is based on the fact that hydrophilic cells adhere more to hydrophilic surfaces, whereas, hydrophobic cells adhere more to hydrophobic surfaces. Mei et al. (1993) and Rosenberg (1981) reported that in case of bacteria more adhesion of the cells to polystyrene indicated more hydrophobicity of the cell surface.

The adhesion of conidia from 1st subculture was higher at all the three pH tested, indicating more surface hydrophobicity than 40th subculture conidia. The density of adhered conidia from 1st subculture was highest at pH 7.0, decreasing to an intermediate level at pH 5.0 and displaying poor adhesion at pH 3.0, similar trend was also observed for 40th subculture (Fig. 4.6).

This may be due to dehydration of proteins of the conidial wall at high ionic strength through a salting out effect, reducing the adsorption on to low energy surfaces (Rosenberg, 1984).

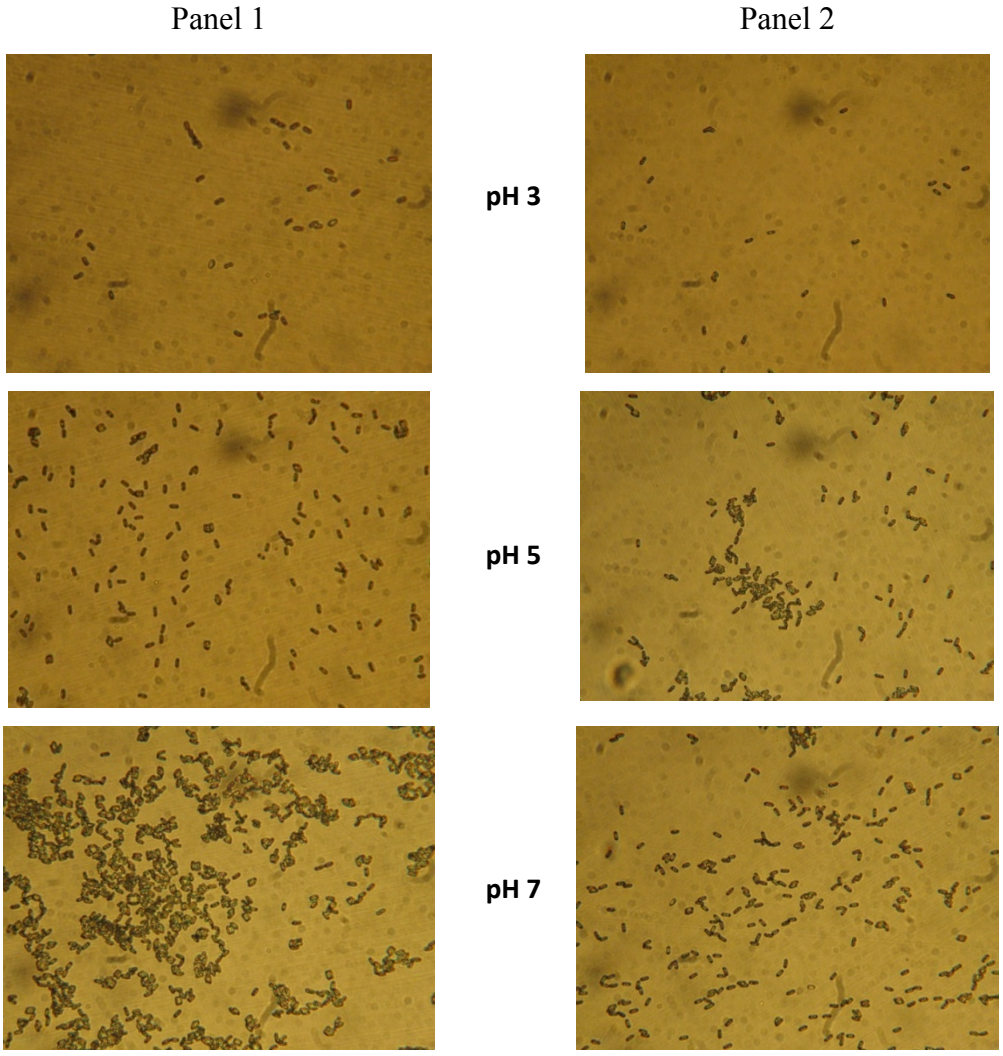


Fig. 4.6 Microscopic images (×400) of *M. anisopliae* adhered to polystyrene petri dish. Conidia from 1st (panel 1) and 40th (panel 2) subculture were suspended in phosphate buffer at pH 3.0, 5.0 and 7.0.

4.4.3 Microbial Adhesion To Hydrocarbon Assay (MATH assay)

MATH assay is based on the affinity of conidia for non-polar solvent with van der waals interactions, for which conidia are partitioned between two immiscible solutions (e.g. water and hexadecane). Cells with hydrophobic index (HI) >0.7 were considered hydrophobic (HI = no. of cells in the organic phase /total no. of cells) (Holder et al, 2007). The conidia of 1st subculture showed 0.81 HI and 40th subculture HI was 0.78. Surfaces of conidia from both the subcultures were hydrophobic, repeated subcuturing may have resulted in the marginal decrease in hydrophobicity/HI as observed for 40th subculture.

4.4.4 Contact angle measurement

According to Reithinger et al. (1997), when the contact angle is <90º, the surface is considered as hydrophilic while for hydrophobic surface the angle is >90º. In other words, more the contact angle more is the hydrophobic surface. The contact angle was measured by a Digi drop technique on the surface of *M. anisopliae* sporulated growth. *M. anisopliae* 1st subculture conidia showed higher contact angle (130º) than 40th subculture (119º) sporulated growth (Fig. 4.7). Reithinger et al. (1997) suggested that measurement of contact angle between water and conidial surface exclusively measured the hydrophobicity of conidial surface and was not affected by other physiochemical and structural factors involved in microbial adhesion. The results suggested that the surface of conidia from 1st subculture was more hydrophobic than 40th subculture.

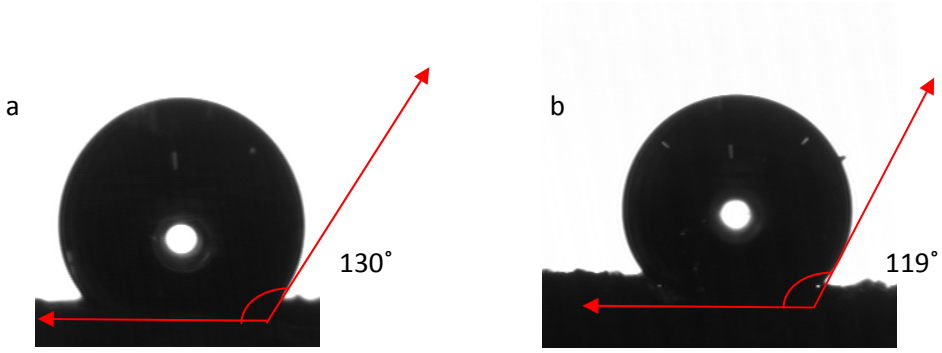


Fig. 4.7 Contact angle measurements of water droplets placed on (a) 1st subculture and (b) 40th subculture *M. anisopliae* sporulated growth.

4.4.5 Zeta potential measurement

Zeta potential measures the electrostatic properties and surface charges of the conidia (Wilson et al., 2001, Strevett and Chen, 2003). Dunlap et al. (2005) suggested that surface charges of conidia could influence the performance of conidial formulations by interacting with formulation adjuvant. Therefore, quantification of surface charge and understanding the factors influencing surface charge could prove very useful to improve adhesion. Shah et al. (2007) had reported change in zeta potential of conidia after repeated subculturing.

The zeta potential of conidia from 1st and 40th subculture did not show any significant difference (Table 4.1). Zeta potential values for conidia from 1st and 40th subcultures indicated their moderate stability.

Table 4.1 Effect of repeated *in vitro* sub-culturing on surface properties of *M. anisopliae* conidia

Surface properties	1st subculture conidia	40th subculture conidia
ST_{50} (hours)	2.30	2.70
HI	0.81 ± 0.01	0.78 ± 0.01
Adhesion to polystyrene		
pH 3	++	+
pH 5	+++	++
(pH 7)	++++	+++
CA (˚)	130° ± 0.70	119° ± 0.53
ZP (mV)	-36 ±1.91	-35±0.76
Mortality		
% Mortality (1×10^7/ml)	97	86
LT_{50}*	3.3	5.6

HI: hydrophobic Index; CA: Contact angle; ZP: Zeta potential; ST_{50}-Settling time of conidia

4.4.6 Atomic force microscopy (AFM)

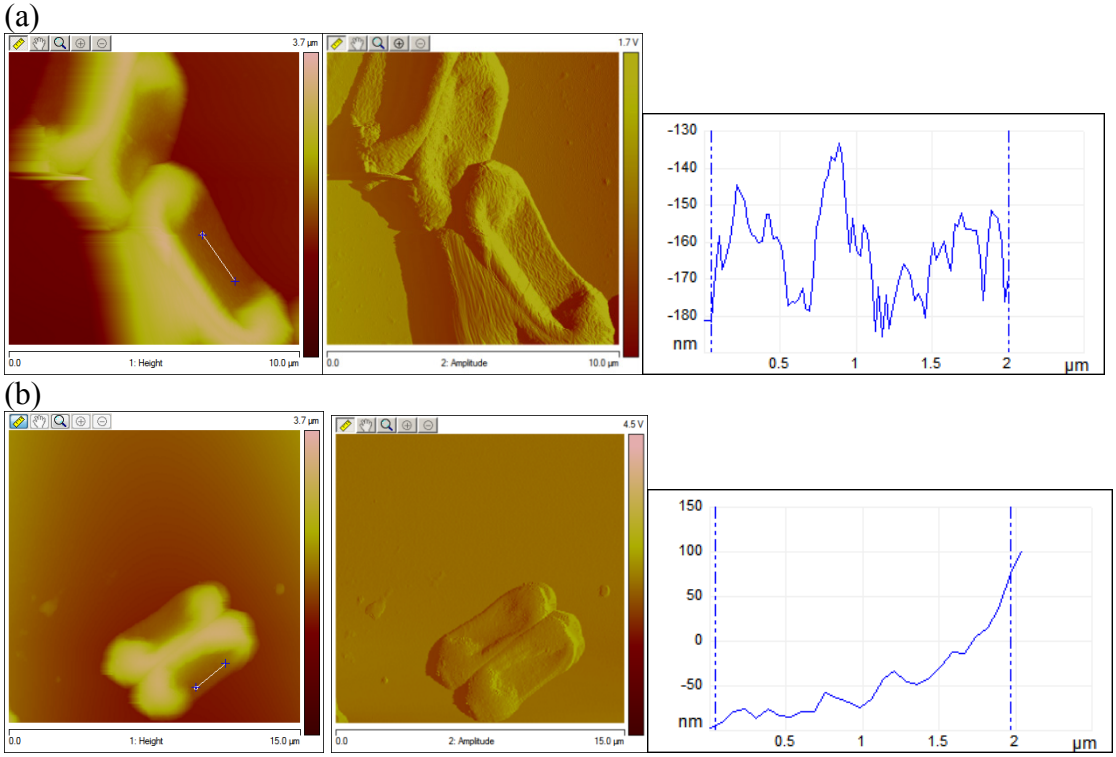
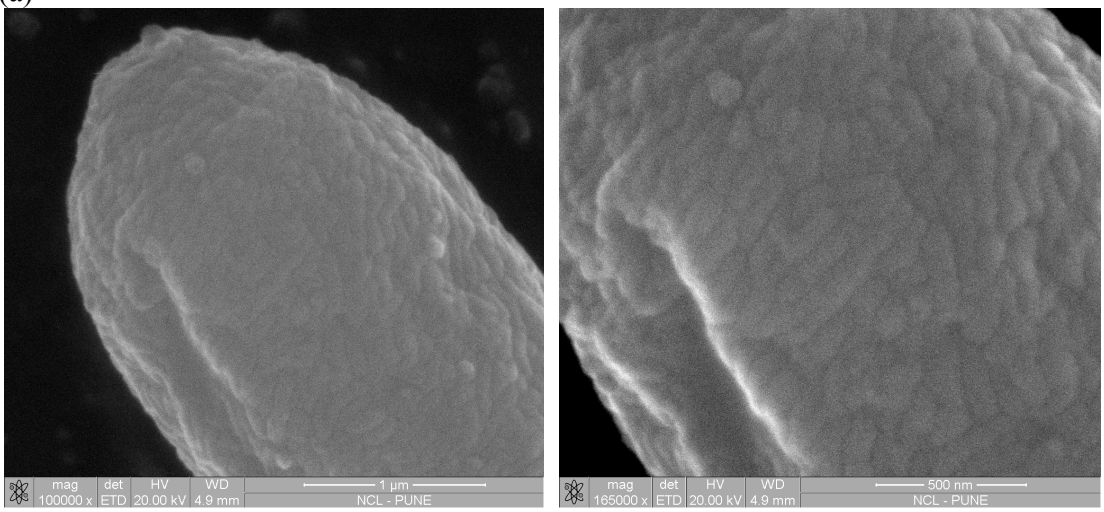


Fig. 4.8 Atomic force micrographs of *M. anisopliae* subcultures: Height and amplitude image with surface profile of (a) 1st subculture conidia and (b) 40th subculture conidia

The detailed surface topological features of *M. anisopliae* conidia were studied by AFM. The surface of *M. anisopliae* conidia was found to be rough (Table 4.1), wherein 1st subculture conidia showed 21% more roughness RMS (nm) than 40th subculture conidia. Roughness is the direct measure of cell surface hydrophobicity. Higher the roughness, higher is the hydrophobicity (Holder et al., 2007). The roughness of *M. anisopliae* conidia may be attributed to the presence of fascicle bundles presumably composed of assembled hydrophobins rodlet, which were clearly visible on the surface of conidia (Fig. 4.8).

4.4.7 Scanning (SEM) and transmission electron microscopy (TEM)

(a)



(b)

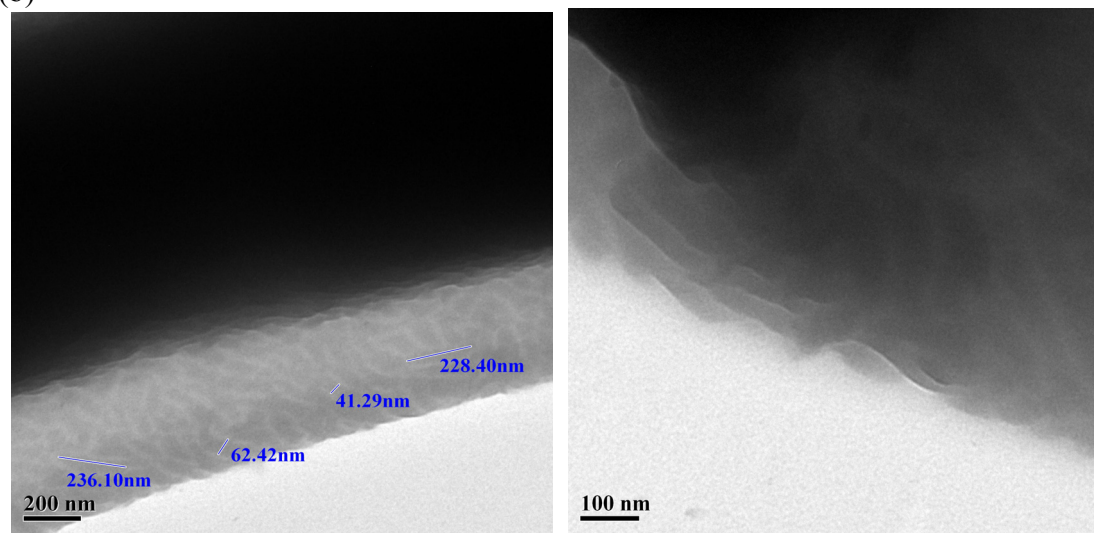


Fig. 4.9 (a) Scanning electron microscopy images (SEM) and (b)Transmission electron microscopy images (TEM) of 1st subculture conidia surface of *M. anisopliae*

The surface morphology of the *M. anisopliae* conidia from 1st subculture was examined by electron microscopy. The study revealed the presence of rodlet layer on conidial surface with bundles like arrangement (Fig. 4.9). Paris et al. (2003) showed the presence of rodlet layers on the conidial surface of *A. nidulans* and *A. niger,* which was mainly due to insoluble protein complexes. In *B. bassiana*, hyd1 and hyd2 hydrophobins were found to contribute to the formation of rodlet layer on conidial surface (Zhang et al. 2011). Both these hydrophobins are known to play distinct physiological roles in the processes that include rodlet layer formation, conidial thermostability and mediating cell surface properties that contribute to virulence to the insects.

4.4.8 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and identification of hydrophobins

The surface proteins were extracted from *M. anisopliae* 1st and 40th sub-culture conidia as described in Material and Methods (section 2.14.1) and subjected to SDS-PAGE. Two bands, corresponding to ~11kDa and ~16kDa, were resolved on 15% gel when stained with comassie brilliant blue (Fig. 4.10 a). The bands were eluted from gel and separated bands were visualised by silver staining (4.10 b). Paris

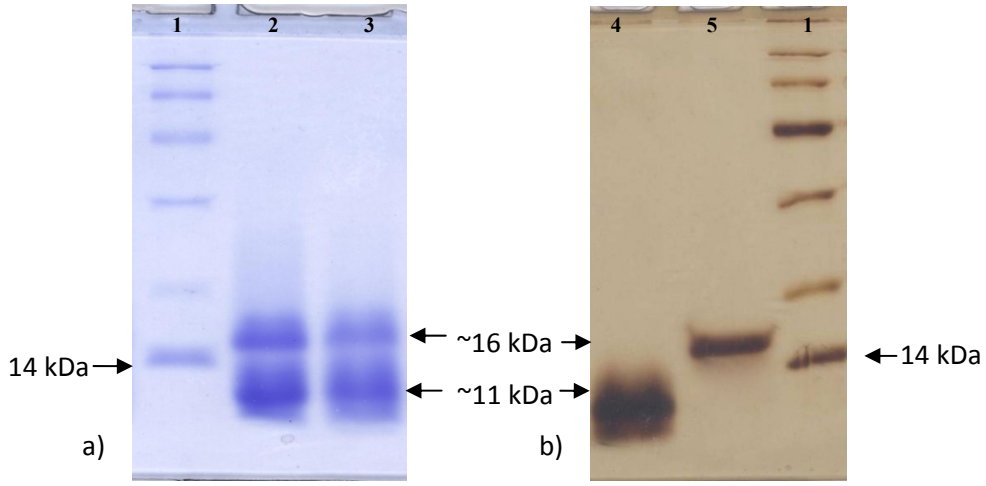


Fig. 4.10 Analysis of hydroflouric acid-soluble protein of *M. anisopliae* 1st and 40th subcultures conidia. Separation of proteins on 15% SDS PAGE and (a) staining with comassie brilliant blue; Lane 1: Protein Marker; Lane 2: 1st subculture conidia; Lane 3: 40th subculture conidia; (b) staining with silver nitrate; Lane 4: lower band of hydrophobin; Lane 5: Upper band of hydrophobin

et al. (2003) reported the presence of 14kDa and 16kDa proteins as insoluble complexes on the conidial surface of *A. nidulans* and *A. niger*. Similarly, Ying and Fang, (2004) were recognized three bands of 12kDa, 15kDa and 17kDa as hydrophobins from *B. bassiana* conidial surface protein extracts.

Table 4.2 Coverage of hydrophobin peptides of *M. anisopliae* with hydrophobins from other fungi

S. no.	Organism	Accession no.	% Coverage	Peptides
1	*Aspergillus lentulus*	Q58XI6	96.63	9
2	*Neosartorya fischeri*	Q0WY30	95.65	7
3	*Aspergillus fumigatiaffinis*	F8VB86	87.30	3
4	*Neosartorya quadricincta*	O59998	76.52	8
5	*Aspergillus duricaulis*	O59985	73.91	7
6	*Neosartorya fumigata*	O59994	62.60	5
7	*Neosartorya fennelliae*	O59992	54.78	7
8	*Neosartorya spathulata*	O60000	46.08	5
9	*Metarhizium anisopliae*	Q0PIV9	10.20	4
10	*Beauveria bassiana*	A4L7H2	9.55	1

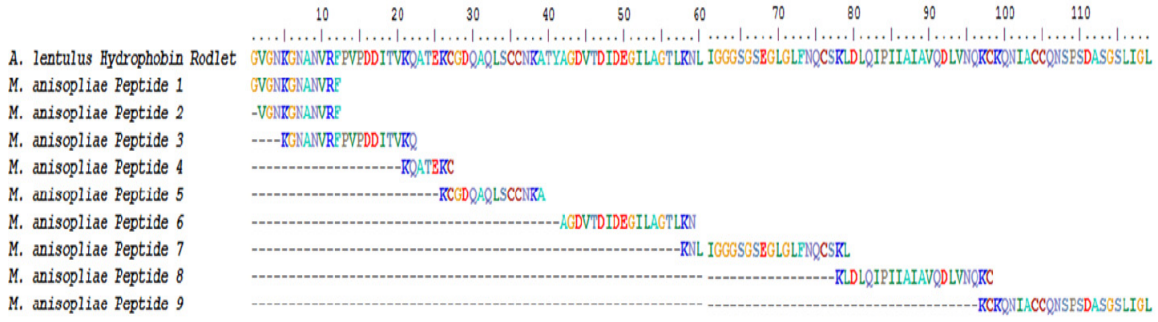


Fig. 4.11 The alignment of peptide sequences of *M. anisopliae* with *A. lentulus*

MALDI-TOF and LC-MS analysis of the peptide fragments liberated by tryptic digestion of the eluted protein bands (~11kDa and ~16kDa) were carried out for protein identification. The spectra of both the protein bands were matched to peptides that could be correlated to hydrophobins from other fungi (Table 4.2). The alignment of peptide sequences obtained by LC-MS and MALDI analysis showed

significant identity (96%) with hydrophobin rodlet of *Aspergillus lentulus* (Fig. 4.11), thereby confirming the proteins as hydrophobins. Bayry et al., (2012) reported that hydrophobins were low molecular mass proteins (<20 kDa) secreted by fungi. In *B. bassiana*, the non-specific hydrophobic interactions between the fungal spore coat hydrophobins and the insect epicuticle was involved in establishing the pathogenicity (Zhang et al., 2011).

4.5 Evaluation of conidial adhesion to the insect cuticle

The adhesion of *M. anisopliae* conidia (1st and 40th subculture) to insect cuticle was determined by soaking the conidia inoculated larvae (1×10^7 conidia/ml) in dichloromethane (DCM) as described by Ment et al. (2010). The adhesion of 1st subculture conidia ($2.16\pm0.33\times10^6$ conidia/larvae) to the insect cuticle was found to be higher than the 40th subculture conidia ($1.90\pm0.28\times10^6$ conidia/larva). As no difference was observed in germination efficiency of conidia from 1st and 40th sub-culture, the difference in adhesion of conidia along with more appressoria formation may be responsible for higher virulence of 1st subculture conidia than 40th subculture. The difference in adhesion may be linked to changes in the surface properties of conidia, which may arise due to repeated *in vitro* subculturing. Shah et al. (2007) reported that there was 12.5% and 22% decrease in conidial attachment from 1st to 9th subcultures of *M. anisopliae* V275 and *M. anisopliae* V245, respectively.

4.6 Insect bioassay

Third instar larvae of *H. armigera* were used to assess the change, if any, in virulence of *M. anisopliae* M34412 due to repeated sub-culturing. Bioassays with 1st subculture conidia showed higher mortality and faster killing as compared to conidia from 40th sub-culture (Table 4.1). *M. anisopliae* 1st sub-culture conidia were found to be more virulent with LT_{50} 3.3 d as compared to 40th subculture conidia with LT_{50} 5.6 d. In another study with *M. anisopliae* M34412, Nahar et al. (2008) reported that for *Ceratovacuna lanigera*, the first sub-culture conidia recorded lowest LT_{50} value (4.4 d) while it was 4.9 d and 5.6 d for the 20th and 40th subcultures, respectively. Shah et al. (2007) reported that successive subculturing of two strains of *M. anisopliae* up to 9th subculture on nutrient-rich media resulted in rapid changes in the surface

properties of conidia corresponding with a decline in virulence. LT_{50} values reduced from 3.74 to 4.46 d for one strain and 4.79 to 5.57 d for the other strain.

In the present study, the effect of repeated *in vitro* subculturing on the conidial surface properties, thereby on fungus-insect interactions and virulence of fungus to insect has been reported. Nahar et al. (2008) correlated the attenuation in virulence of *M. anisopliae* M34412 to decreased appressorium formation and cuticle degrading enzyme production. Results from present investigations suggests that additionally, the adhesion of *M. anisopliae* conidia to the insect cuticle, which is the first step in fungus-insect interaction, was also affected due to repeated *in vitro* subculturing. The surface hydrophobicity of 1^{st} subculture conidia was higher than 40^{th} subculture, resulted in increased adherence of 1^{st} subculture conidia to insect cuticle and thereby increased mycosis. The surface of conidia was hydrophobic in nature due to the presence of rodlet layers of proteins, identified as hydrophobins. The surface roughness could be directly correlated to the presence of hydrophobin rodlet layers. Higher adhesion, surface roughness and hydrophobicity suggested the role of hydrophobins in establishing the contact of conidia with the insect cuticle. Thus, the surface properties of conidia were found to be an important factor in fungus-insect interaction.

Earlier reports supported these observations, suggesting hydrophobic forces were responsible for the passive, nonspecific adhesion of *M. anisopliae* conidia to insect cuticle (Boucias et al, 1988). The present study showed that repeated sub-culturing causes attenuation of virulence by altering several factors responsible for *M. anisopliae* pathogenicity, more specifically CDE activities, appressorium formation and also surface properties of conidia. The knowledge of surface physico-chemical properties of *M. anisopliae* conidial surface thus provided a basis for predicting how these conidia would interact with their insect hosts and their host's environment.

C. Degradation of cuticular hydrocarbons of *H. armigera* during *M. anisopliae* pathogenesis

Unlike other microorganisms which need to be ingested for infection to occur, entomopathogenic fungi act by attachment of the conidia to the host cuticle, followed by germination and appressorium formation. This is followed by penetration of the

host cuticle which involves mechanical pressure of the forming hyphae and enzymatic degradation of major cuticular components, i.e., proteins, chitin and lipids (Fargues, 1984; Gillespie et al., 2000). It has been reported that epicuticular layer is heterogeneous and strongly influences conidial germination, resulting in differential susceptibility of various insect species to a fungal pathogen (Thompson, 1973; Boucias and Pendland., 1988; El-Sayed et al., 1991; Wang et al., 2005). Boucias and Latge (1988) proposed that insect epicuticular lipids might be important in fungal attachment to the host cuticle. Thus, the composition of the host cuticle is an important criterion for establishing the efficacy of entomopathogenic fungi.

The epicuticular layer comprises a complex mixture of non-polar lipids (usually long chain hydrocarbons, wax esters, fatty alcohols and free or esterified fatty acids) which play a major role in preventing desiccation, chemical communication and acts as a barrier affecting the penetration of chemicals, including pesticides (Blomquist et al., 1987; Blomquist and Vogt, 2003). Insect hydrocarbons are the most abundant components and usually comprise a mixture of *n*-alkanes, *n*-alkenes and methyl branched alkanes. *n*-alkanes, usually in the chain length range C_{21}-C_{35}, are the predominant hydrocarbons. *n*-alkenes are present as isomeric mixtures with the position of the double bond variable. The methyl branched alkanes are with one to four methyl groups inserted in the terminal or internal positions as mono, di-, tri- or tetramethyl alkanes. Mono and dimethyl alkanes are the mostly found in insects (Pedrini et al., 2007).

For conidial germination, a carbon source is necessary (Smith and Grula, 1981). While chitin and certain fatty acids were shown to be efficiently utilized (Ferron, 1985), early studies also indicated the utilization of hydrocarbons as well as sterols and glycerols (Smith and Grula, 1981). The ability of entomopathogenic fungi to degrade epicuticular hydrocarbons of insects and to use them for energy production and incorporation into cellular components were first demonstrated by Napolitano and Juáirez (1997) and Crespo et al. (2000) where they demonstrated peroxisome proliferation in alkane grown cultures of *B. bassiana*. In a subsequent study, Pedrini et al. (2006) reported the induction of peroxisomal catalase in alkane grown *B. bassiana* cultures. Comprehensive studies on insect cuticular lipids have shown that hydrocarbons are widespread in all insect orders (Blomquist and Dillwith, 1985).

In the present study, in order to understand the role of cuticular hydrocarbons during fungus-insect interaction, the identification and quantitative determination of hydrocarbon components of 3rd instar larvae of *H. armigera* at different time intervals during the infection process of *M. anisopliae* over a period of 120 h was done.

4.7 Degradation of cuticular hydrocarbons during pathogenesis

4.7.1 Qualitative analysis of cuticular hydrocarbons

GC-MS analysis of hydrocarbons of 3rd instar larvae of *H. armigera* over a period of 120 h following exposure to conidia of *M. anisopliae* revealed the quantitative changes. Over 60 major hydrocarbon components were characterised (Fig. 4.12; Table 4.3). Kovats' retention index for all the components was calculated using the formula –

$$KI = 100z + 100\left(\frac{\log t_s - \log t_z}{\log t_{z+1} - \log t_z}\right)$$

Where: ts, tz and tz+1 - net to retention time of the compound of interest, and standards with z and (z+1) carbon numbers of *n*-alkanes.

Hydrocarbon components consisted of a homologous series of *n*-alkanes (C_{14}-C_{33}), isomeric series of internally branched monomethyl alkanes, a series of internally branched dimethyl alkanes, tetra-methyl alkanes and alkenes. Within the *n*-alkane portion of the hydrocarbon profile in this species, odd-chain *n*-alkanes were quantitavely present in higher amounts, the predominant one being C_{27} (heptacosane) (Table 4.4).

Heptacosane has been reported to be the major alkane in the pupae of a related species, *H. virescens* where they account for about 11% of the total alkane (Coudron and Nelson, 1978) and also in the larvae of *H. virescens* and *H. zea* (Nelson and Buckner, 1995). In the larvae of the sugarcane borer, *Diatraea saccharalis*, the *n*-alkanes account for approximately 6.7% and comprise C_{23}-C_{33} carbon atoms with C_{27} being the major component (Girotti et al., 2012).

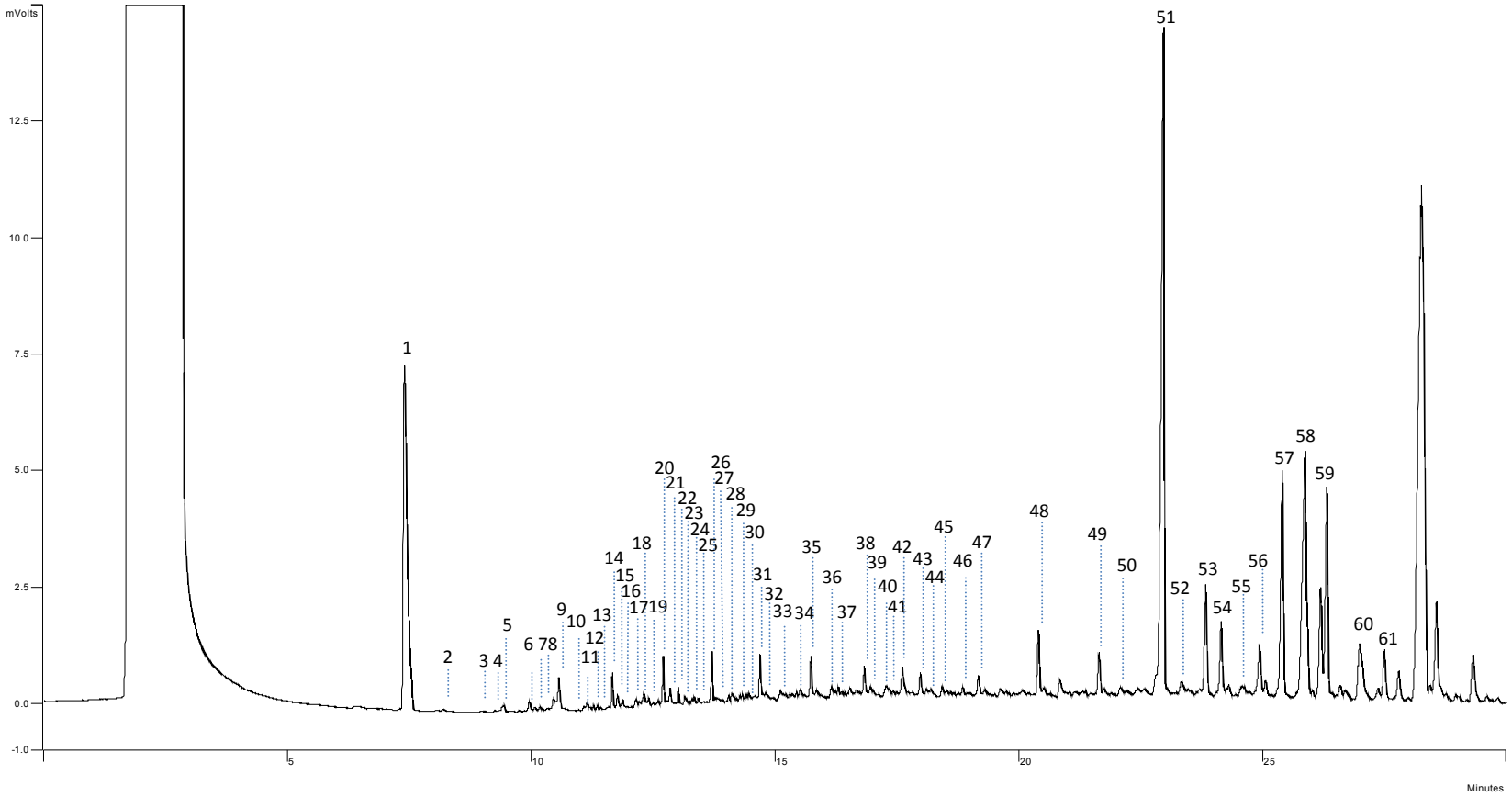


Fig. 4.12 GC-MS chromatogram of cuticular hydrocarbons from 3rd instar larvae of *H. armigera*. Peaks labelled with numbers

Table 4.3 Analysis of hydrocarbons of 3rd instar larvae of *H. armigera*

S.no.	KI	Hydrocarbons	S.no.	KI	Hydrocarbons
1	1326	1-Bromodecane	32	2019	unknown
2	1400	Tetradecane	33	2059	2-methyl eicosane
3	1462	2 methyl tetradecane	34	2074	3-methyl eicosane
4	1472	3 methyl tetradecane	35	2100	Heneicosane
5	1500	Pentadecane	36	2138	5-methyl heneicosane
6	1561	4-methyl pentadecane	37	2154	3-methyl heneicosane
7	1567	2-methyl pentadecane	38	2200	Docosane
8	1574	3-methyl pentadecane	39	2074	5-methyl docosane
9	1600	Hexadecane	40	2220	2-methyl docosane
10	1649	7-methyl hexadecane	41	2220	2,4-dimethyl docosane
11	1655	4-methyl hexadecane	42	2230	(z)-9-Tricosene
12	1666	2-methyl hexadecane	43	2300	Tricosane
13	1627	3-methyl hexadecane	44	2311	6-methyl tricosane
14	1700	Heptadecane	45	2319	5-methyl tricosane
15	1711	2,6,10,14-tetramethyl heptadecane	46	2350	3-methyl tricosane
16	1747	2,6-dimethyl heptadecane	47	2400	Tetracosane
17	1760	4-methyl heptadecane	48	2500	Pentacosane
18	1766	2 methyl heptadecane	49	2600	Hexacosane
19	1774	3-methyl heptadecane	50	2687	1-docosanol
20	1800	Octadecane	51	2696	Heptacosane
21	1845	2-methyl octadecane	52	2752	9-methyl heptacosane
22	1854	5 methyl octadecane	53	2761	4,8-dimethyl heptacosane
23	1860	4-methyl octadecane	54	2783	Octacosane
24	1865	2-methyl octadecane	55	2836	2-methyl octacosane
25	1873	3-methyl octadecane	56	2837	unknown
26	1900	Nonadecane	57	2867	Nonacosane
27	1906	9-methyl octadecane	58	2897	unknown
28	1960	4-methyl nonadecane	59	2918	Tricontane
29	1964	2-methyl nonadecane	60	2999	1-Docosane
30	1973	3-methyl nonadecane	61	3000	9-Hexosene
31	2000	Eicosane			

Table 4.4 Amount of *n*-alkanes in hydrocarbons from 3rd instar larvae of *H. armigera*

n-alkane	Amount (Ng/g of *H. armigera*)
C_{16} Hexadecane	1350.89
C_{17} Heptadecane	1742.26
C_{18} Octadecane	757.25
C_{19} Nonadecane	446.11
C_{20} Eicosane	585.56
C_{21} Henicosan	629.55
C_{22} Docosane	503.62
C_{23} Tricosane	862.93
C_{24} Tetracosane	1533.77
C_{25} Pentacosane	5596.03
C_{26} Hexacosane	2458.65
C_{27} Heptacosane	43831.92
C_{28} Octacosane	3571.34
C_{29} Nonacosane	12100.07
C_{30} Triacontane	569.84
C_{31} Hentriacontane	1705.54
C_{32} Dotriacontane	293.83
C_{33} Tritricosane	430.39

4.7.2 Degradation of cuticular hydrocarbons of 3rd instar larvae of *H. armigera* by *M. anisopliae*

Larvae of *H. armigera* when exposed to conidia of *M. anisopliae* showed gradual reduction in the amounts of hydrocarbons over a period of 120 h as evidenced by a reduction in the peak heights of the GC profiles (Fig. 4.13). A gradual decrease in the amount of cuticular hydrocarbons from 100% at 0 h to 21.8% at the end of 120 h was observed (Fig. 4.14 a) which was largely due to the reduction in the amount of *n*-alkanes (from 100% at 0 h to 12.04% at the end of 120 h) (Fig. 4.14 b). The results suggested utilization of *n*-alkanes as the primary source of carbon for growth by *M. anisopliae*. In contrast, there was no apparent decrease in the amounts of branched alkanes (Fig. 4.14 c).

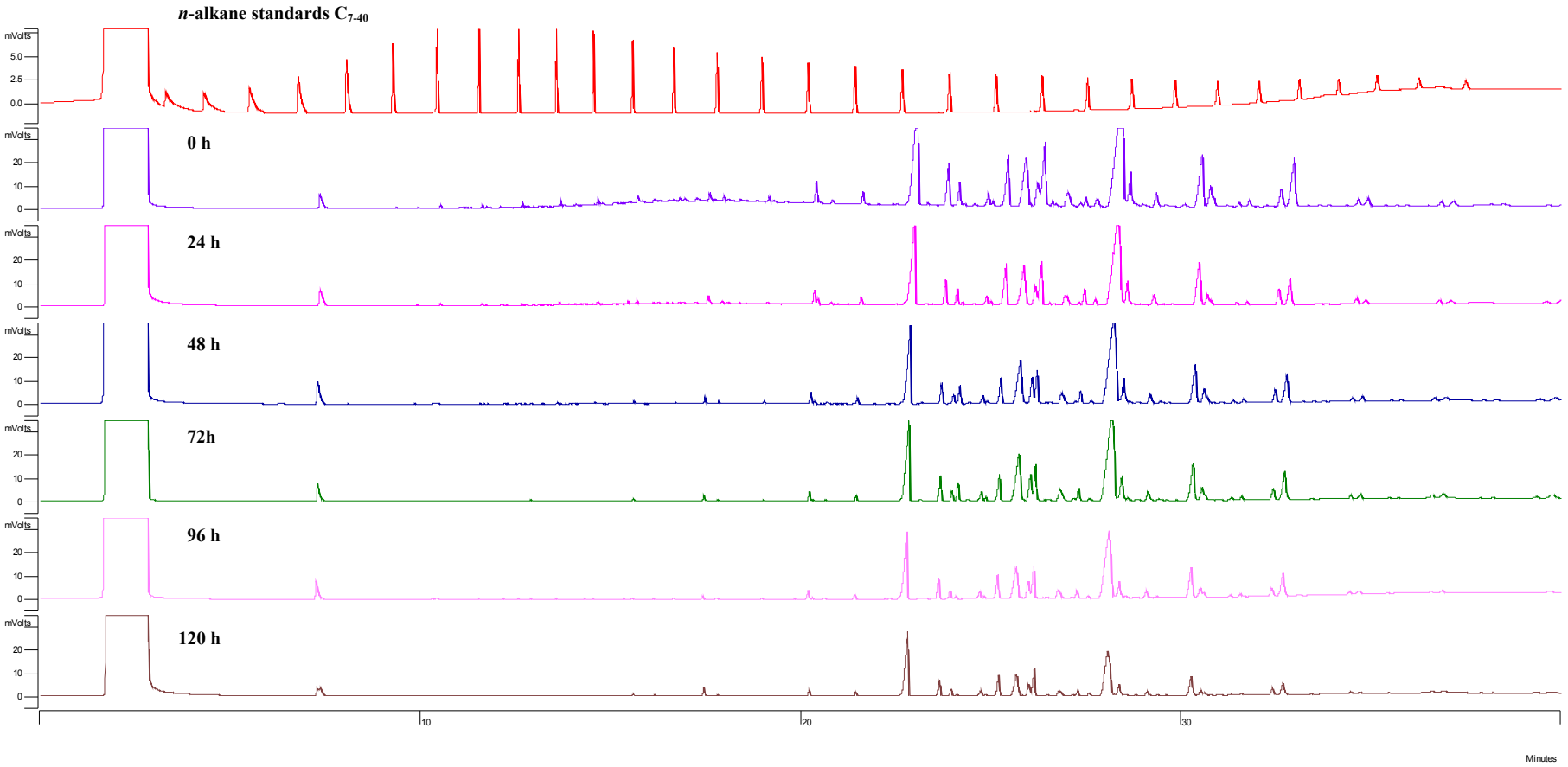


Fig. 4.13 GC profiles of cuticular hydrocarbons of 3rd instar *H. armigera* larvae exposed to conidia of *M. anisopliae* (1×10^7 conidia/ml)

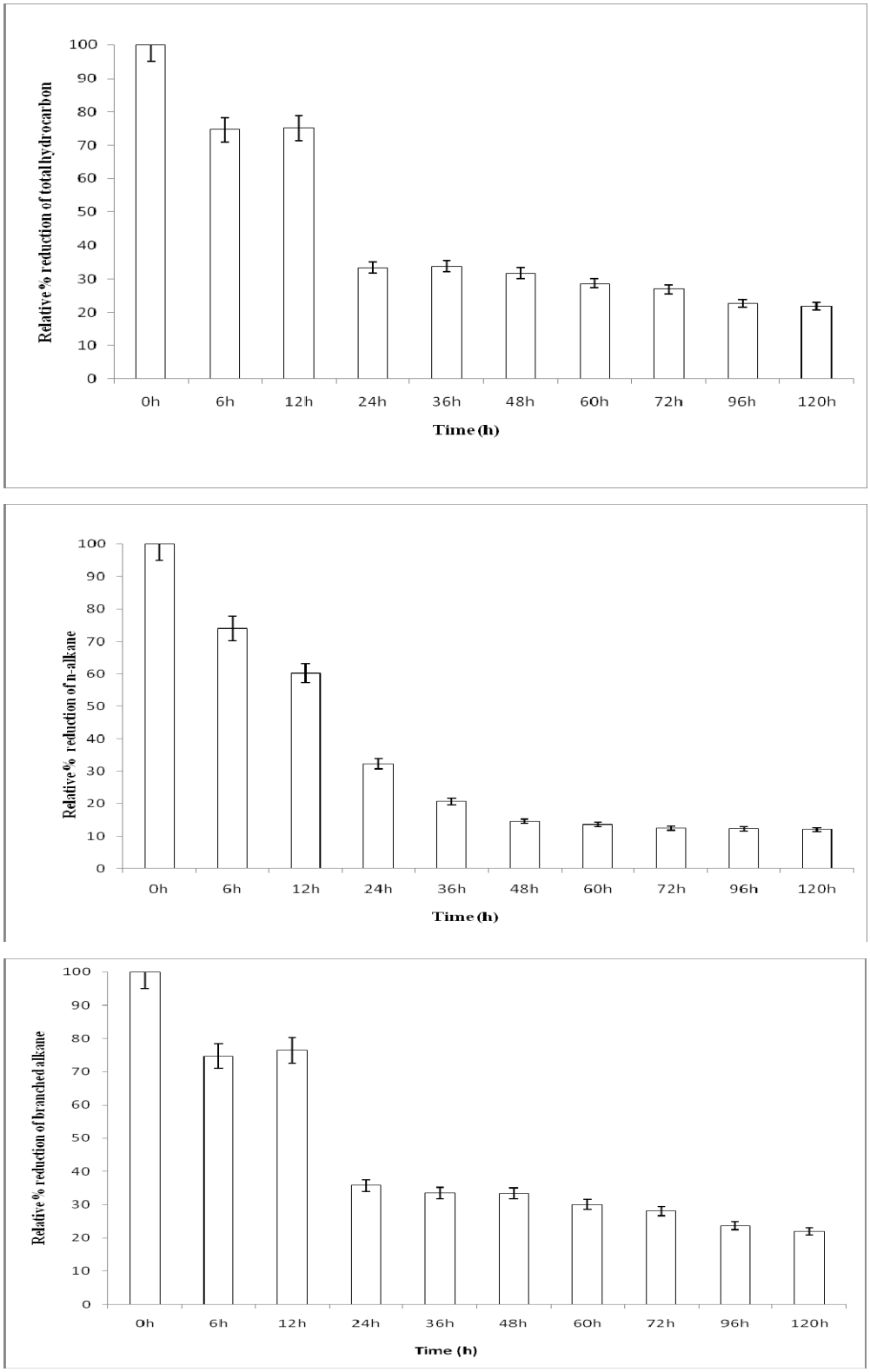
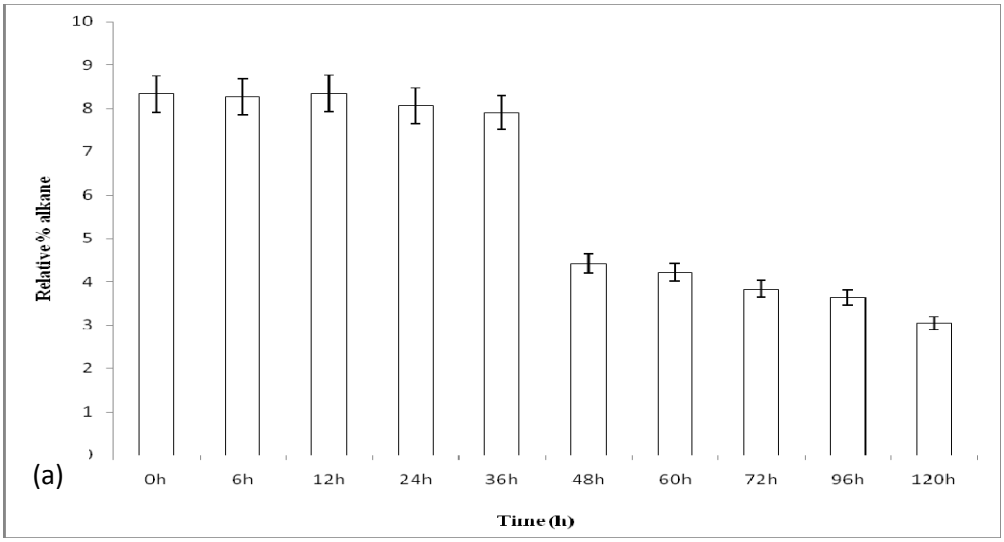


Fig. 4.14 Relative percent hydrocarbons at different time intervals from 3rd instar larvae of *H. armigera* exposed to *M. anisopliae* conidia a) Percent total hydrocarbon; b) Percent total *n*-alkanes and c) Percent total branched *n*-alkanes



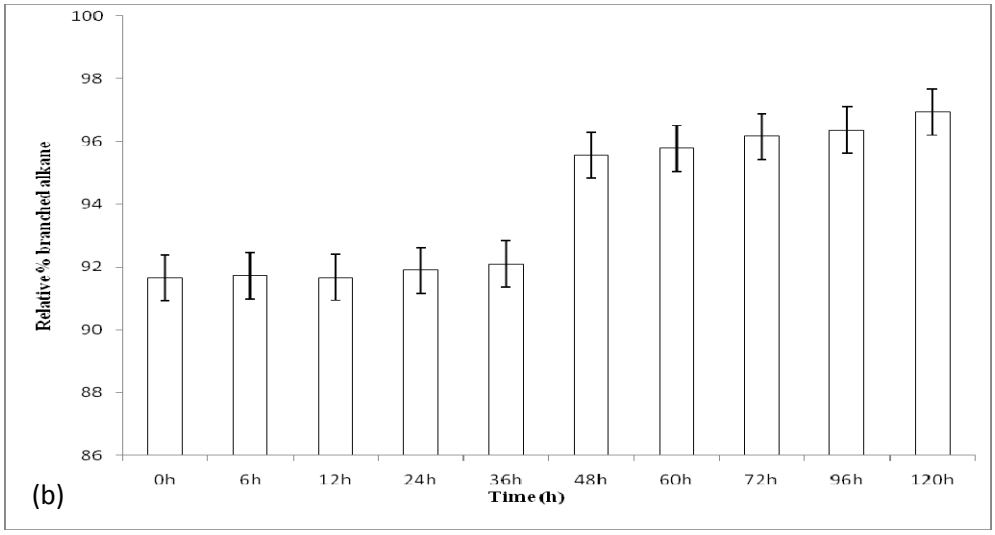


Fig. 4.15 (a) Relative percent *n*-alkanes and (b) branched *n*-alkanes at different time intervals with respect to hydrocarbons of *H. armigera* exposed to *M. anisopliae* conidia

When comparisons were made between percent alkanes with respect to the percent total hydrocarbons, percent degradation was more apparent. *n*-alkanes represented 8.33% of the total hydrocarbons at 0 h and remain at this level till 36 h following which there was a drastic reduction to 4.42% at 48 h. At the end of 120 h, the percent alkanes with respect to the total hydrocarbons were reduced further to 3.04% (Fig. 4.15 a). In contrast, the branched alkanes remained constant around 91% until 36 h following which they increased marginally to 96% (Fig. 4.15 b). Not much is known about synthesis of hydrocarbons in insects. Some early studies have demonstrated that hydrocarbons are transported by haemolymph lipophorin (Fichera

and Brenner, 1982; Juairez and Brenner, 1985). Crespo et al. (2000) and Napolitano and Juairez (1997) reported the degradation of heptacosane, pentacosane, octacosane and methyl branched nonacosane with chain lengths similar to the major hydrocarbon components of the bean weevil, *Acanthoscelides obtectus* which were incorporated into the fungal lipids.

The study highlights the degradation of hydrocarbons of *H. armigera* larvae by *M. anisopliae* thereby suggesting possible chemical recognition at the host cuticular surface during the initial stages of fungal infection. Further work is needed to identify the enzymes involved and to determine if there is any correlation between strain virulence and enzyme activities.

D. Production and surface properties of *M. anisopliae* blastospores

Blastospores are another infective propagule produced by *M. anisopliae* during submerged fermentation. The liquid culture production of *Metarhizium* blastospores and its use for the control of various insects have been evaluated in numerous studies (Stephan and Zimmermann, 1997; Kleespies and Zimmermann, 1998; Fargues et al., 2002; Leland et al., 2005). Optimization of *M. anisopliae* M34412 blastospore production, characterization of surface properties of blastospores and its possible correlation with pathogenecity is discussed in subsequent sections.

4.8 Blastospores production

4.8.1 Selection of liquid media for blastospores production

Each strain has different responses to the composition of media, pH and temperature; therefore it is necessary to optimize the media for higher blastospores production in shorter fermentation time. *M. anisopliae* blastospore production was optimized with respect to media components, pH and inoculum concentration.

Blastospores production of *M. anisopliae* by submerged fermentation using agro-industrial residues and semi synthetic media were optimized with respect to substrate and fermentation conditions in 1 L Erlenmeyer flasks. Blastospores production was carried out by inoculating *M. anisopliae* mycelium (10% v/v) in 10

different media. The highest production of blastospores was obtained in Admek medium (containing Corn steep liquor) after 96 h (Table 4.5). Kleespies and Zimmerman (1992) and Fargues et al., (2002) have used the corn steep liquor media for increased blastospores production.

Table 4.5 Production of *M. anisopliae* blastospores in different liquid media

Medium	Blastospore count/ml
YPG	$4.0\pm0.24\times10^{6}$
YPG 5%	$5.36\pm0.20\times10^{6}$
YPG 10%	$2.6\pm0.14\times10^{6}$
Jackson	$3\pm0.16\times10^{7}$
Paris	$1.6\pm0.20\times10^{5}$
Kondryatiev	$5\pm0.13\times10^{6}$
Admek medium (with corn steep liquor)	$1.2\pm0.07\times10^{8}$
Corn steep solid containing medium	$5\pm0.22\times10^{7}$
CSL Molases	$1\pm0.13\times10^{8}$
Czapek-dox	$0.3\pm0.16\times10^{6}$

*Flask were incubated at 28˚C, 180 rpm for 96 h

4.8.2 Effect of initial pH on blastospores production

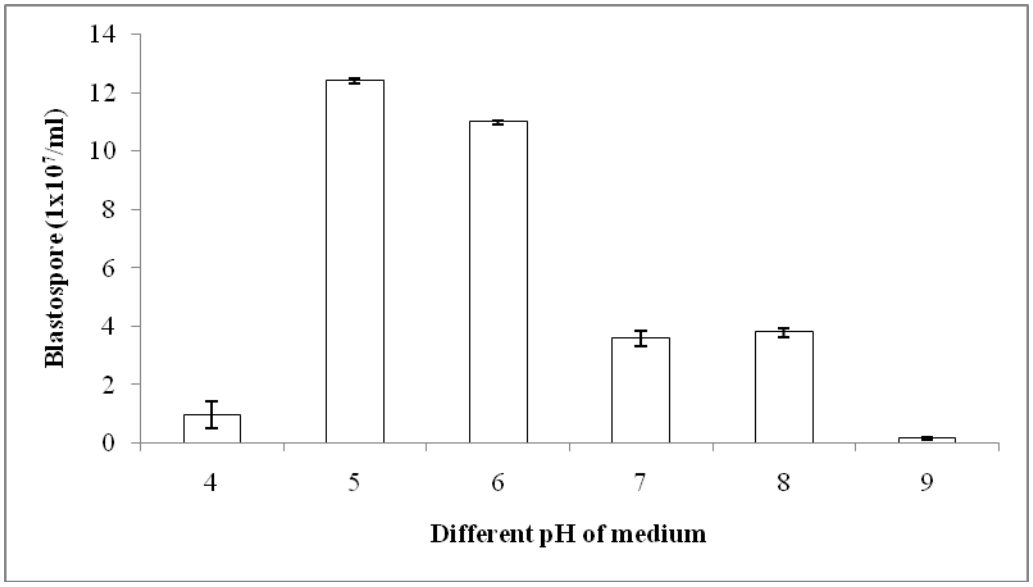


Fig. 4.16 Effect of initial pH on blastospores production of *M. anisopliae*

Effect of initial pH of the medium on blastospores production was studied in Admek medium at pH ranging from 4 to 9 (Fig. 4.16). The pH 5 was found to be optimum for blastospores production and the yield was 1.2×10^8 blastospores/ml. The final pH of the medium decreased to pH 3.5 after 96 h. Fang et al. (2008) also reported higher yield of *M. anisopliae* blastospores at pH < 5.

4.8.3 Effect of conidia, mycelia and blastospores as inocula on the production of blastospores

Different morphological forms of *M. anisopliae* such as, mycelium, blastospores and conidia were used as inocula for blastospores production. The mycelial inoculum gave maximum yield of blastospores as compared to other inocula used (Table 4.6).

Table 4.6 Effect of conidia, mycelia and blastospores as inocula on the production of *M. anisopliae* blastospores

Inoculum	Blastospores produced /ml
Conidia (1×10^7 /100 ml)	$0.8\pm0.010\times10^8$
Blastospore (1×10^7/100 ml)	$0.8\pm0.15\times10^8$
Mycelium (10% v/v)	$1.2\pm0.07\times10^8$

While Jackson et al. (2003) reported that conidial inoculums gave maximum blastospores production. Similarly, Ypsilos and Magan (2004) also showed higher blastospores production using conidial inoculum in *M. anisopliae.* However, in the present investigation lower yield of blastospores was obtained with conidial inoculum as compared to mycelial inoculum. Therefore, for further studies, mycelium was used as an inoculum.

4.8.4 Effect of inoculum concentration

The effect of inoculum concentration (2-50% (v/v)) was studied in Admek medium. The 10% (v/v) mycelial inoculum concentration was found to be optimum for the production of blastospores with 1.2×10^8 blastospores/ml yield in 96 h (Fig. 4.17).

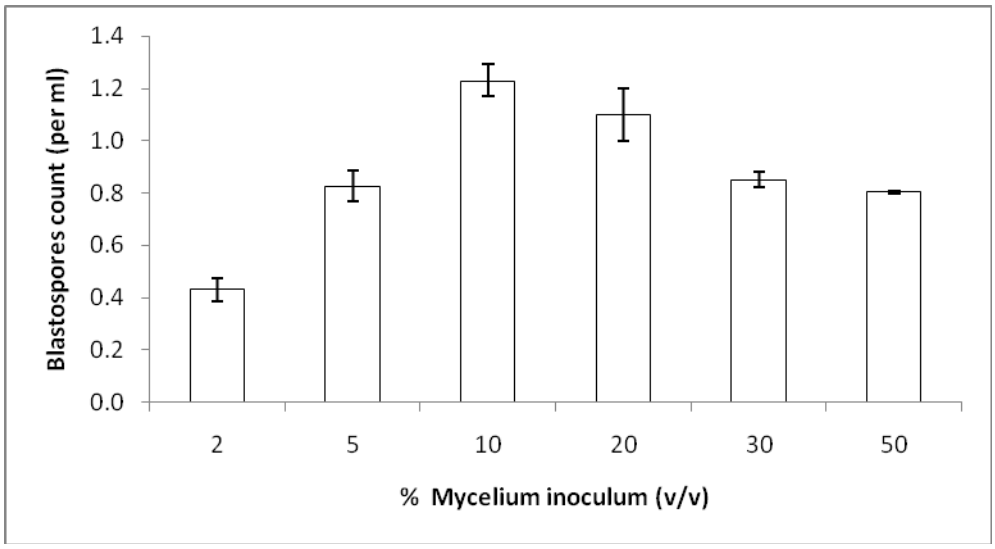


Fig. 4.17 Effect of mycelium inoculum concentration on blastospore production

4.8.5 Blastospores production under optimized conditions

The blastospores production was monitored under optimised conditions for 120 h. The initial lag period (0-48 h) was followed by the steady increase in blastospores production (up to 96 h). The highest blastospores production (1.2×10^8 blastospores/ml) was obtained after 96 h. On further incubation, reduction in blastospores count was observed. The steady decrease in pH from 5 to 3.5 was observed during the incubation period (Fig. 4.18).

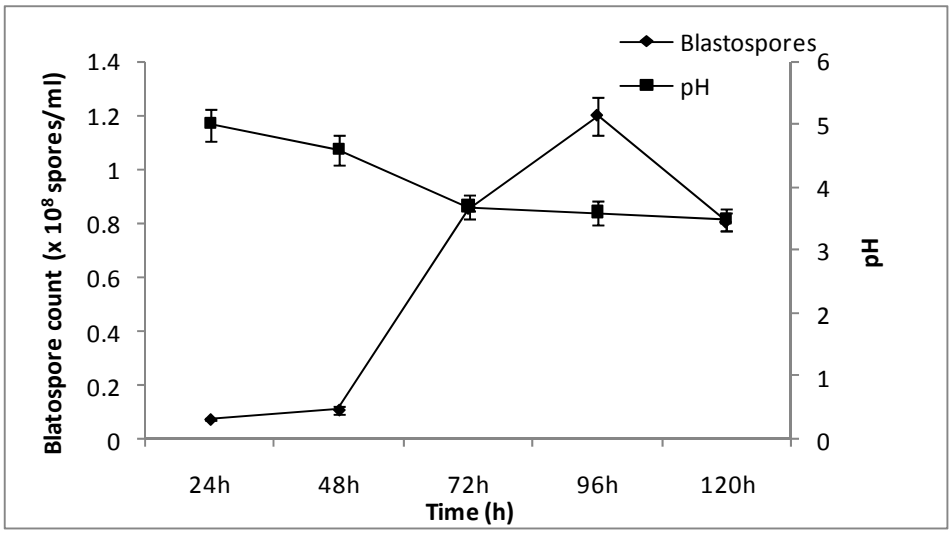


Fig. 4.18 Production of blastospores of *M. anisopliae* M34412 in Admek medium and concomitant change in pH

The growth of *M. anisopliae* under submerged fermentation for the production of blastospores was found to be strain-dependent (Vega et al., 2003). Ypsilos and Magan (2004) reported maximum blastospores production in *M. anisopliae* after 72 h, while Jackson and Jaronski (2009) observed relatively higher blastospores production (1.6×10^8 blastospores/ml) from *M. anisopliae* after 96 h.

4.9 Germination of blastospores and appressorium formation

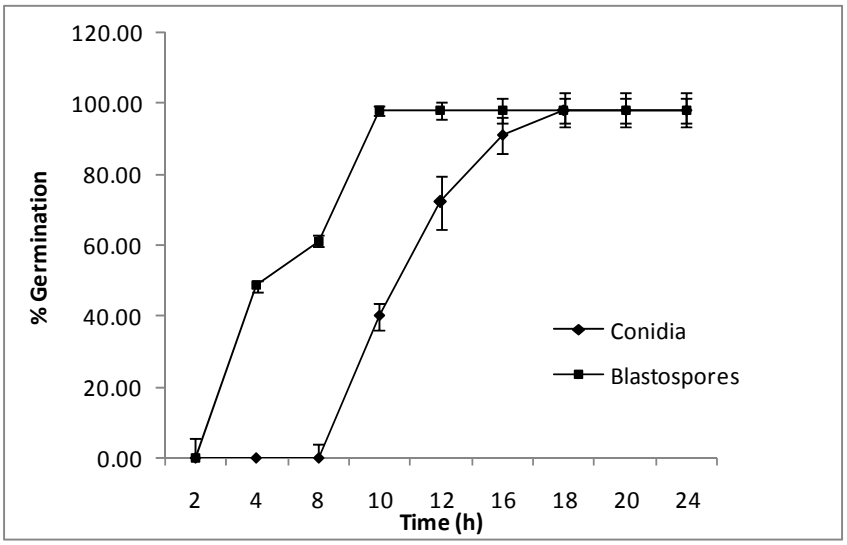


Fig. 4.19 Germination of conidia and blastospores of *M. anisopliae* on PDA agar

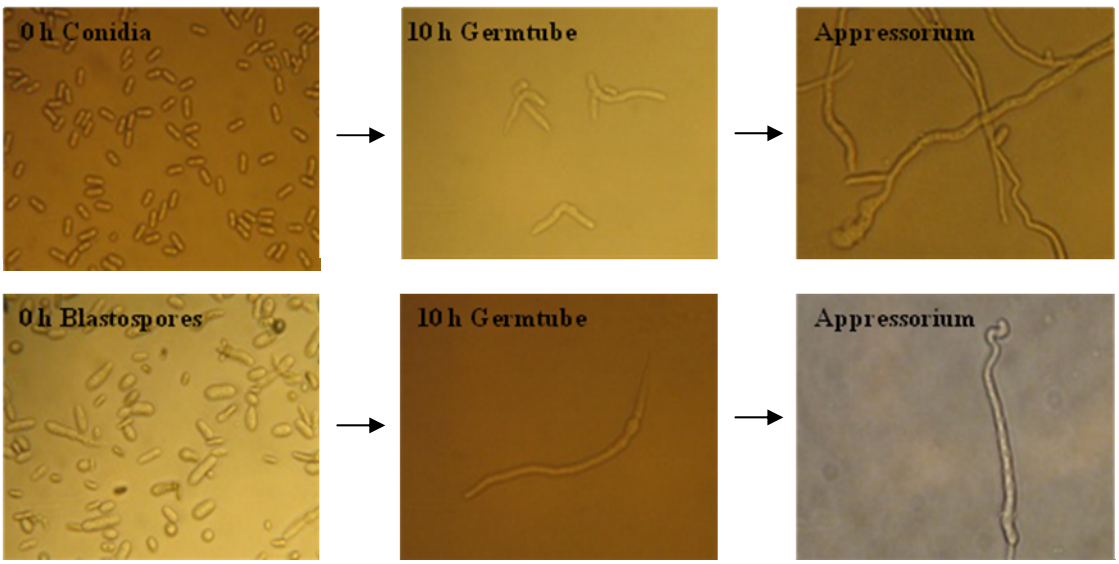


Fig. 4.20 Germtube and appressorium formation from conidia and blastospores of *M. anisopliae* (400x)

Germination rate of fungal propagule plays an important role in virulence. Germination rate of conidia and blastospores of *M. anisopliae* was tested. It was

observed that more than 90% of blastospores germinated after 6 h incubation on PDA agar, while *M. anisopliae* conidia required 16 h incubation to achieve more than 90% germination (Fig. 4.19). Similar observations were reported in *P. fumosoroseus* (Jackson et al. 2003). The germination and appressorium formation pattern of *M. anisopliae* conidia and blastospores are shown in Fig. 4.20.

4.10 Production of cuticle degrading enzyme from blastospores

The production of cuticle degrading enzymes *viz*, chitinase, chitosanase, protease and lipase were studied in a chitin medium. The levels of enzymes were obtained by using blastospore as inoculum were 3.96×10^{-3}U/ml chtinase; 31.44×10^{-3} U/ml chitosanase; 3.41 U/ml protease and 0.996 U/ml lipase. The levels of all these enzymes were not significantly different from that obtained with conidial inoculum.

4.11 Surface properties of blastospores

Characterization of surface properties of the blastospores was done and the results were compared with the conidia (Table 4.7). The slower rate of sedimentation indicated smooth and hydrophilic nature of blastospores surface as compared to that of conidia. Furthermore, HI obtained by MATH assay and contact angle data confirmed the hydrophilic nature of blastospores surface (Fig. 4.21). The AFM analysis showed the presence of smoother surface of blastospores than conidia. The bundles of rodlet layers were not observed indicated the absence of hydrophobins on the surface of blastospores (Fig. 4.22). It was further confirmed by SDS-PAGE analysis, where no bands of hydrophobins were detected as seen in case of conidia. Similar observations were reported in *B. bassiana* (Holder and Keyhani, 2005; Holder et al., 2007).

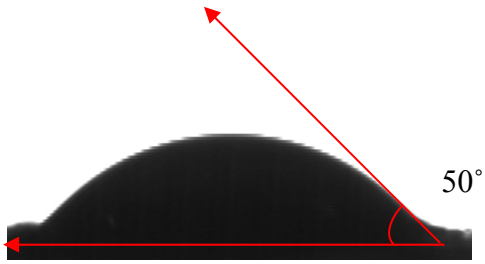


Fig. 4.21 Contact angle of water on Blastospores mat

Table 4.7 Comparison of surface properties of *M. anisopliae* blastospores with conidia and mortality of *H. armigera*

***M. anisopliae* form**	**HI**	**CA (°)**	**ST_{50} (h)**	**ZP (mV)**	**Roughness RMS (nm)**	**% Mortality**	**LT_{50} (d)**
Conidia (1st subculture)	0.81±0.01	130±0.70	2.3±0.2	-36±1.91	33±3.20	97	3.3
Blastospores	0.30±0.01	50±0.17	3±0.1	-19±0.90	13.4±0.96	97	3.1

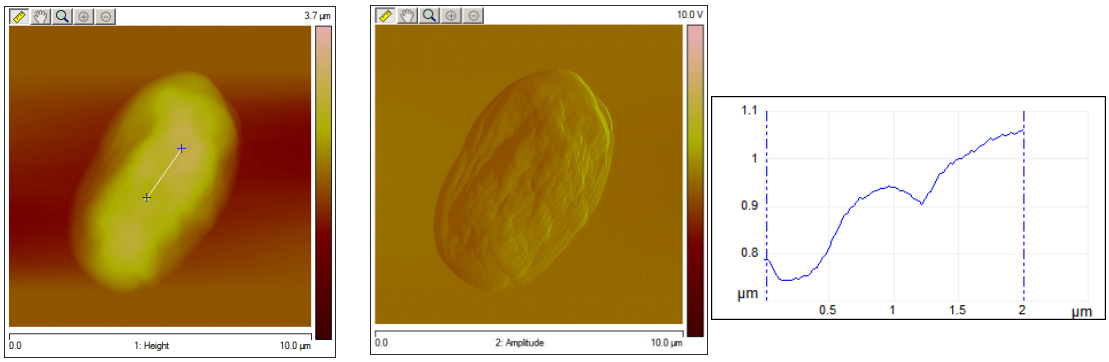


Fig. 4.22 AFM height and amplitude images of *M. anisopliae* blastospres with mean height profile

Though the surface properties of conidia and blastospores were found to be significantly different, no difference was seen in their infectivity. The data suggests that in addition to the surface properties of fungal propagules, the properties of insect cuticle may also be playing an important role in fungus-insect interaction. Hydrophobins, the protein which were found to play crucial role in adhesion of conidia to insect cuticle, were completely absent on the surface of blastospores. However, once adhered to insect cuticle, blastospores were found to germinate and grow faster leading to mycosis. It suggests that the mechanism of interaction of blastospores with insect cuticle may be different than the mechanism by which conidia interacts.

4.12 Stability of *M. anisopliae* conidia and blastospores

The effect of different storage temperatures on stability of conidia and blastospores was studied. The conidia were found to be stable at all temperatures even

after 2 weeks. Whereas, the viability of blastospores decreased with increase in the the storage temperature and ~90% viability was lost at ambient temperature (Table 4.8).

Table 4.8 Temperature stability of conidia and blastospores of *M. anisopliae*

	% Viability					
	Conidia			**Blastospores**		
Time/Temp (ºC)	-80ºC	4ºC	28ºC	-80ºC	4ºC	28ºC
Freshly harvested	>95	>95	>95	>95	>95	>95
1 Week	>95	>95	>95	90	82	25
2 Week	>95	>95	>95	90	75	10

Munaoz et al. (1995) reported the effect of difference in hydrophobicity of propagules on viability of *Trichoderma harzianum*. They showed that aerially produced conidia were highly hydrophobic and retained their viability on longer storage as compared to blastospores. The aerial conidia were found to be highly resistant to environmental variations due to the presence of hydrophobins on their surfaces. Also, the outer hydrophobic layers were thought to protect the conidia from dehydration in the environment (Boucias et al., 1988).

4.13 Effect of fungal propagules on mortality of *H. armigera*

The mortality of *H. armigera* was same with blastospores and conidia. However, the LT_{50} was found to be lower for blastospores (Table 4.7). In some cases, for instance *B. bassiana* (Lane et al., 1991; Robert and Sweeny, 1982), blastospores had a lower virulence in comparison to aerially produced conidia. Whereas, for *V. lecanii,* equal efficacy was observed (Hall, 1981).

The information may be useful for understanding interactions with formulation adjuvant and for choosing formulation conditions to improve adhesion. However, the properties of cuticle of *H. armigera*, another dimension of the fungus-insect interaction, needs to be comprehensively studied.

Chapter 5

Host range of *Metarhizium anisopliae* and its evaluation against *Aedes aegypti*

Insects and other pests can badly damage plant roots, stems, leaves, flowers and seeds and cause immense to agriculture and to the ecosystem. For example, *S. litura* (Fabricius) (Lepidoptera: Noctuidae), Rice Cutworm, earlier known to be a sporadic pest, has emerged into major polyphagous pest in the recent past. This pest has been reported to attack a wide range of crops (about 40 species of plants) in Indian sub-continent (Chari and Patel 1983).

Similarly, Grape mealy bug, *M. hirsutus* (Pink) (Hemiptera: Pseudococcidae) is causing severe crop losses (30-70%), is a serious economic threat to agriculture in India (Ujjan and Shahzad, 2007).

M. anisopliae, an entomopathogenic biocontrol agent, has the potential of controlling several insect pests (Zimmerman, 2007). *M. anisopliae* was shown to be effective against a broad host range of insects belonging to the orders of Lepidoptera (*H. armigera*, *S. litura*), Hemiptera (*C. lanigera*, *M. hirsutus*), Coleoptera (Beetles) and Diptera (Mosquitoes and flies) (Butt et al., 1992; Kulkarni et al., 2008; Sree et al., 2008; Farenhorst et al., 2010).

Vector borne diseases like malaria, filariasis, dengue and chikungunya caused by mosquito genera such as *Anopheles stephensi, Culex quinquifasciatus* and *Aedes aegypti* have a significant impact on human health. An important criterion for successful transmission of disease is the longevity of insect vectors and thus control efforts are focused on methods that cause a rapid reduction in adult survival (Smith and Mackenzie, 2004). Efforts to control such insect vectors largely depend on the use of chemical pesticides like organochlorines, organophosphates and pyrethroids with a quick knock-down effect (Das and Amalraj, 1997; Farenhorst et al., 2010). Among the bacterial species evaluated for vector control, *Bt.* var. *israelensis* and *Bacillus sphaericus* were used as larvicides exerting control by ingestion (Fillinger et al., 2008; Geissbuhler et al., 2009). The use of entomopathogenic fungi, such as *M. anisopliae* which are effective by contact of conidia have been demonstrated too. Furthermore, *B. bassiana* and *M. anisopliae* are reported to be highly effective against insecticide resistant mosquitoes such as *An. Gambiae* (Kikankie et al., 2010; Farenhorst et al., 2009; Howard et al., 2010).

In case of mosquito larvae that proliferate in aqueous milieu, fungal conidia adherence to the cuticle as well as ingestion followed by germination and subsequent disruption of the gut chitin with the aid of chitinolytic enzymes have been reported

(Lacey et al., 1988; Silva et al., 2008; Seye et al., 2009). In addition to the lethal effect of entomopathogenic fungi on mosquitoes, they were also reported to cause reduction of vectorial capacity, feeding, fecundity, flight or dispersal capacity as well as predator escape responses (Scholte et al., 2006; Thomas and Read, 2007; Blanford et al., 2009; Read et al., 2009). Therefore, it was thought worthwhile to evaluate the potential of the three most effective strains identified in present study for the control of larvae and adults of *Ae. aegypti*.

Results and Discussion

5.1 Bioassays with different insect pests

Studies were conducted with *M. anisopliae* to exploit their potential for controlling the insect pests of different order. The laboratory bioassay showed that the *M. anisopliae* were found to be pathogenic to all the 4 insect species tested (Table 5.1). However, there was a variation in their infectivity against different pest. While considering host specificity, among the virulence parameters of the fungal isolate, mortality could be taken as the main trait. In the present study, *M. anisopliae* were more effective against different populations within *H. armigera*, *S. litura* and *Ae. aegypti*. Therefore, it is not possible to conclude if *M. hirsutus* insect species are in general less susceptible to *M. anisopliae.* The waxy coat of *M. hirsutus* may have affected the adhesion of conidia, essential to initiate infection resulting in less mortality in these insect species. Loc and Chi, (2007) reported that *M. anisopliae* were also effective against diamond black moth (*Plutella xylostella*) and Coconutbeetle (*Brontispa longissima*).

Table 5.1 % Mortality of different insect pests during bioassays with 1×10^7 conidia/ml of *M. anisopliae* (M34412)

Sr. No.	Name of insect species	% Corrected mortality
1.	*H. armigera*	97
2.	*S. litura*	93
3.	*M. hirsutus*	65
4.	*Ae. aegypti*	
	Adult	67
	Larvae	98

5.2 Bioassays with *Ae. aegypti* larvae

Among the 3 *M. anisopliae* strains, M34412 was most effective and virulent particularly to the larvae of *Ae. aegypti*. In the presence of 1×10^7 conidia/ml, 3rd instar larvae of *Ae. aegypti* were susceptible to all the 3 strains of *M. anisopliae* with >95% mortality at the end of 7 d of exposure. At the lowest concentration of 1×10^3 conidia/ml, percent mortality ranged between 10-32% for all the 3 strains (Table 5.2). Percent mortality was significantly higher as compared to control (M34412 - $F_{5, 36}=6.75$, $p<0.0001$; M34311– $F_{5, 36}=7.22$, $p<0.0001$ and M81123 - $F_{5, 36}=5.16$, $p<0.001$). Values of LC_{50} varied from 5.92×10^3 conidia/ml for M34412, 3.49×10^4 conidia/ml for M34311 and 5.12×10^5 conidia/ml for M81123 (Table 5.2). Alves et al. (2002) reported a LC_{50} of 1.97×10^4 conidia/ml for isolate 1037 from the several *M.*

Table 5.2 Cumulative corrected mortality (%) and lethal time (LT_{50}) of *Ae. aegypti* 3rd instar larvae after treatment with different concentrations of conidia of *M. anisopliae* M34412, M34311 and M81123.

***M. anisopliae* Isolate**	**Concentration (conidia/ml)**	**% Mortality**	**LT_{50}**	**LC_{50}**
M34412	1×10^7	98	1.75 (1.11-2.31)	
	1×10^6	97	2.41 (2.02-2.75)	
	1×10^5	93	2.86 (2.04-3.53)	5.92×10^3
	1×10^4	78	3.54 (2.98-4.09)	
	1×10^3	32	6.08 (5.38-6.57)	
M343111	1×10^7	96.66	2.39 (2.01-2.73)	
	1×10^6	95	2.71 (2.53-3.39)	
	1×10^5	93.3	2.98 (2.53-3.39)	3.49×10^4
	1×10^4	68.3	4.46 (3.82-5.29)	
	1×10^3	18.35	7.05 (6.51-7.58)	
M81123	1×10^7	95	2.66 (2.25-3.03)	
	1×10^6	93.3	3.57 (3.09-4.06)	
	1×10^5	81.67	4.75 (4.28-5.25)	5.25×10^5
	1×10^4	75	5.11 (4.64-5.66)	
	1×10^3	10	7.55 (6.93-7.97)	

anisopliae isolates evaluated, while Pereira et al. (2009) reported a LC_{50} of 3.16 $\times 10^5$ conidia/ml and a MST of 5 d for the most virulent strain *M. anisopliae* CG144.

Fig. 5.1 depicts the day-wise mortality of *Ae. aegypti* larvae with *M. anisopliae* strains. The LT_{50} varied from 1.75 d to 3.54 d for M34412, 2.39 d to 4.46 d for M34311 and 2.66 d to 5.11 d for M81123 at the concentrations evaluated, i.e., 1×10^7 to 1×10^3 conidia/ml. Exposure of larvae of *Ae. aegypti* to concentrations of 1×10^6 and 1×10^7 conidia/ml resulted in significantly lowest LT_{50} in strains M34412 and M81123. The concentration of conidia was positively correlated with mortality and maximum increase in mosquito larvae mortality was achieved at a concentration of 1×10^7 conidia/ml.

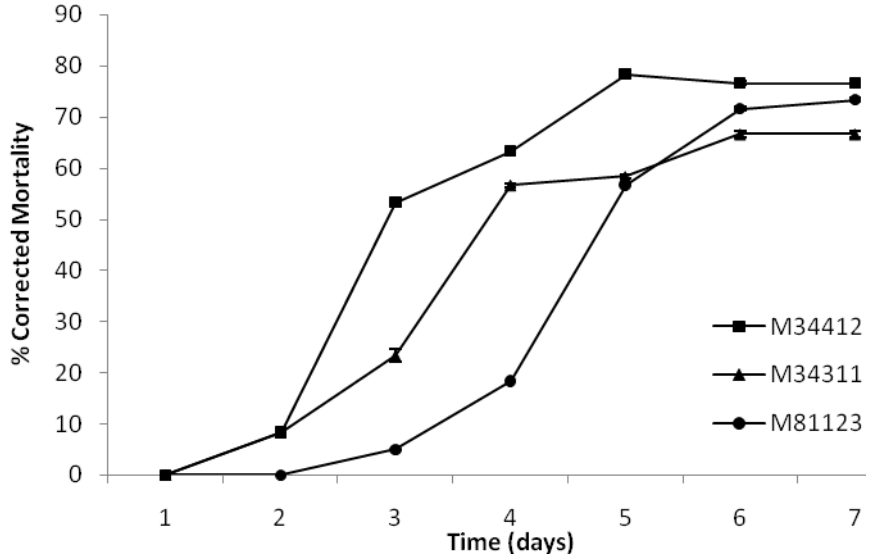


Fig. 5.1 Mean daily mortality rate of larvae of *Ae. aegypti* inoculated with three strains of *M. anisopliae* at $1x10^4$ conidia/ml concentration

Silva et al. (2008) reported 15 out of 80 *M. anisopliae* soil isolates to be highly virulent (>90 % mortality) against 2nd instar larvae of *Ae. aegypti*. While Seye et al. (2009) reported *Aspergillus clavatus* to be the most virulent among the 4 species isolated from the locust, *Oedaleus senegalensis* against larvae of different mosquito species with > 95 % mortality against both *Ae. aegypti* and *Culex quinquefasciatus* and 95% against *Anopheles gambiae.*

5.3 Comparison of 1st subculture and 40th subculture conidia during bioassay with mosquito larvae

Attenuation of virulence of conidia has been observed in nearly all the taxa of entomopathogenic fungi. Attenuated conidia may germinate and infect their hosts marginally slower than non-attenuated conidia, which may partly be due to the lack of the right set of enzymes to facilitate host penetration or change in surface properties leading to less adhesion. The data for percent mortality of *Ae. aegypti* at concentrations 1×10^3, 1×10^5, and 1×10^7 conidia/ml for 1st and 40th subculture conidia are presented in Fig. 5.2. It is evident that though there is >90% mortality at 1×10^5, and 1×10^7 conidia/ml concentrations for both the subcultures, a significant decrease in percent mortaity (~20%) was observed for 40th subculture as compared to 1st subculture at 1×10^3 conidia/ml concentration. Decrease in mortality for 40th subculture may be attributed to decrease in the levels of enzyme activities and concomitant decrease in virulence during *in vitro* repeated transfers from the 1st to 40th sub-culture as demonstrated previously for *H. armigera* (Nahar et al., 2008).

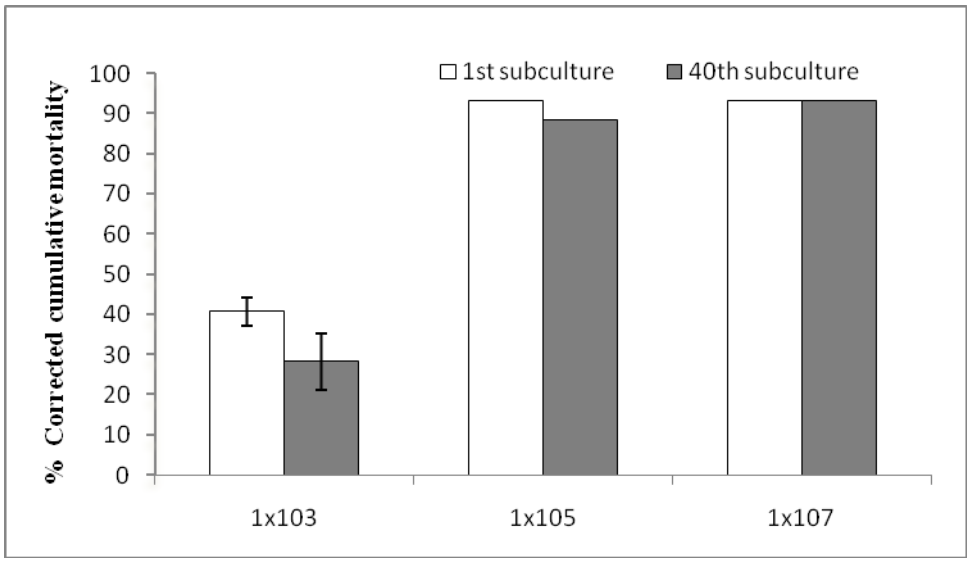


Fig. 5.2 Bioassay of *Ae. aegypti* with *M. anisopliae* (M34412) 1st subculture and 40th subculture conidia at different concentrations

5.4 Combination of *M. anisopliae* and *M. verrucaria* enzyme for larval bioassay

M. verrucaria has been shown to produce a cuticle degrading enzyme (CDE) complex containing chitinase, protease and lipase enzymes with potential application in the control of *Ae. aegypti* (Vyas and Deshpande, 1989). Other fungal products such

as extracellular chitinases from deuteromycetous fungus, *M. verrucaria* and keratinase, an extracellular fungal metabolite from *Trichophyton entagrophytes* were reported to have larvicidal potential against mosquito larvae (Mendonsa et al., 1996; Murugesan et al., 2009). In a recent study, Halder et al. (2012) reported the effect of bacterial chitinolytic enzymes of *Aeromonas hydrophila* on *Cx. quinquifasciatus*, a vector of filariasis.

In present study, the *M. verrucaria* CDE complex was used in different combinations with *M. anisopliae* conidia for bioassay with larvae. The enzyme activities in the CDE complex were - chitinase, 1.5 U/ml; protease, 0.07 U/ml and lipase, 0.415 U/ml. In the bioassay with only *M. verrucaria* CDE complex in terms of chitinase 0.5, 1.0 and 1.5 U/ml enzyme activity, the percent mortality values were 35.83%, 48.84%, and >95% respectively. For treatment with *M. anisopliae* (1×10^3 conidia/ml) singly, the percent mortality was 45.83%. Combination of *M. anisopliae* conidia (1×10^3/ml) with *M. verrucaria* chitinase activity at 0.5 and 1 U/ml showed significant increase in percent mortality (Fig. 5.3).

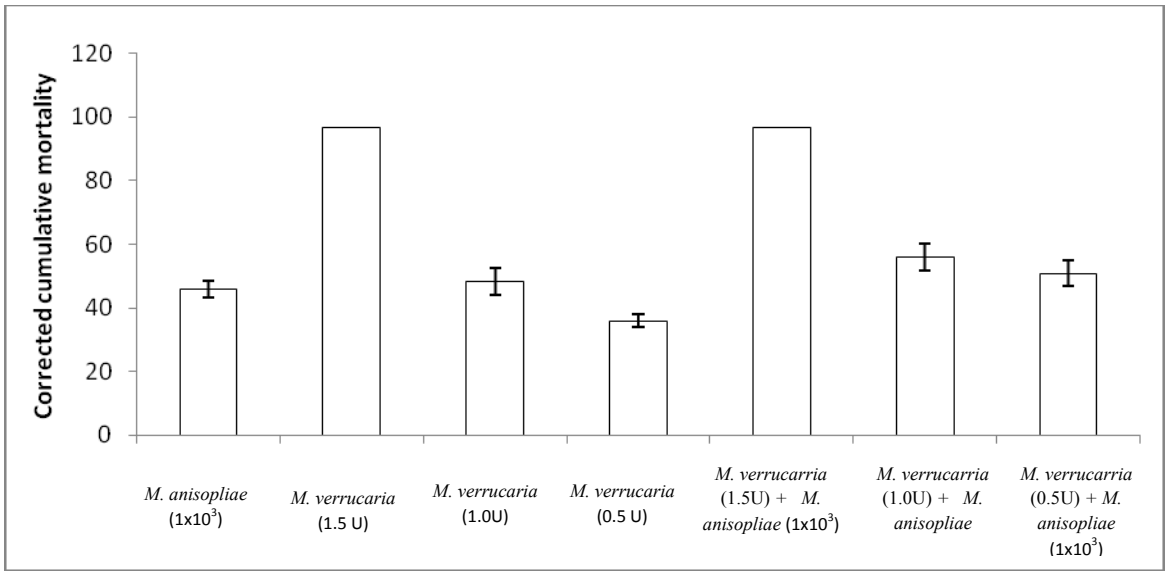


Fig. 5.3 Bioassay of *Ae. aegypti* with *M. anisopliae* conidia (1×10^3conidia/ml) and *M. verrucaria* enzyme complex at different concentrations

5.5 Bioassays with *Ae. aegypti* adults

All the three *M. anisopliae* isolates exhibited significant mortality of adult *Ae. aegypti* after 8 d exposure. At highest concentration (1×10^{10} conidia/ml) tested,

percent mortality values were 93.34 %, 86.7 % and 83.4 % in adults exposed to M34412, M34311 and M81123 strains, respectively. At lower concentrations of 1×10^8 and 1×10^7conidia/ml, it varied from 63.4-76.7% for the 3 strains. At these concentrations, per cent survival was significantly lower than control (M34412 – $F_{4, 35}$=3.87, p<0.01; M34311 – $F_{4, 35}$=3.90, p<0.01; M81123 – $F_{4, 35}$=3.90, p<0.01). The concentrations of conidia that resulted in 50 % mortality (LC_{50}) were 6.92×10^8 conidia/ml for M34412, 5.03×10^9 conidia/ml for 34311 and 8.22×10^9 conidia/ml for M81123 (Table 5.3). Fig. 5.4 depicts the day-wise mortality of *Ae. aegypti* adults with *M. anisopliae* strains.

Table 5.3 Cumulative corrected mortality (%) and lethal time (LT_{50}) of adult females of *Ae. aegypti* after treatment with different concentrations of conidia of *M. anisopliae* M34412, M34311 and M81123

M. anisopliae **Isolate**	**Concentration (conidia/mL)**	**Per cent Mortality**	**LT_{50}**	**LC_{50}**
M34412	1×10^{10}	93.34	3.36 (2.67-3.97)	
	1×10^9	90.00	3.83 (3.15-4.47)	
	1×10^8	76.67	4.52 (3.66-5.47)	6.92×10^8
	1×10^7	66.67	5.40 (4.39-6.99)	
M343111	1×10^{10}	86.67	3.74 (2.95-4.50)	
	1×10^9	83.34	4.08 (3.25-4.93)	
	1×10^8	73.34	4.44 (3.50-5.57)	5.03×10^9
	1×10^7	66.67	5.45 (4.43-7.08)	
M81123	1×10^{10}	83.34	3.98 (3.16-4.80)	
	1×10^9	83.34	4.03 (3.21-4.84)	
	1×10^8	70.00	4.57 (3.56-5.87)	8.22×10^9
	1×10^7	63.33	5.76 (4.80-7.73)	

The LT_{50} varied between 3.36 d to 5.40 d for M34412, 3.74 d to 5.45 d for M34311 and 3.98 d to 5.76 d for M81123 at the concentrations evaluated, i.e., 1×10^{10} to 1×10^7 conidia/ml (Table 5.3).

Studies on entomopathogenic fungi for control of adult *Ae. aegypti* included use of *M. anisopliae* (Scholte et al., 2007; Paula et al., 2008) while for larvae, *Tolypocladium cylidrisporum* (Goettel, 1987; 1988) and *B. bassiana* were reported

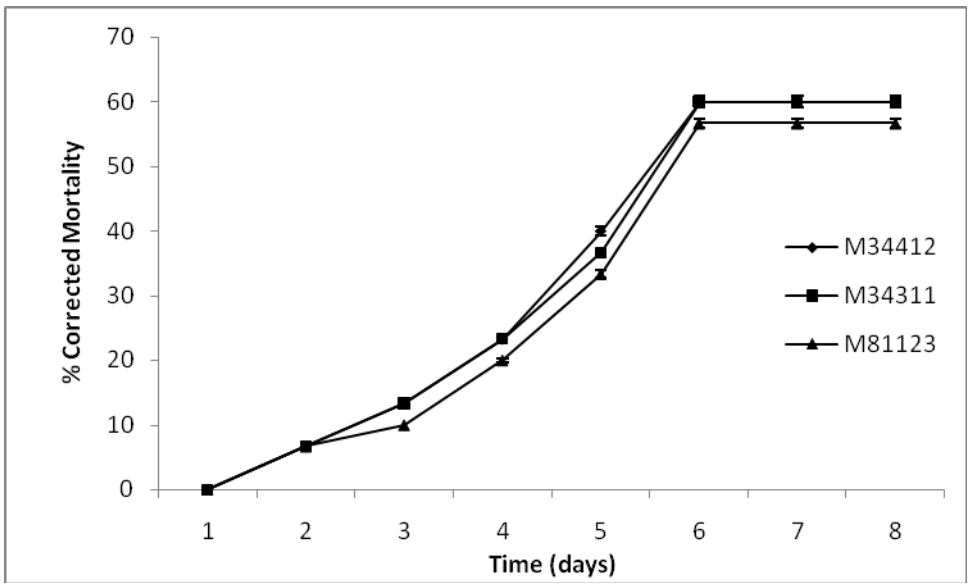


Fig. 5.4 Mean daily mortality rate of adults of *Ae. aegypti* exposed to the three strains of *M. anisopliae* at $1x10^7$ conidia/ml concentration

(Clark et al., 1968; Miranpuri et al., 1991). Furthermore, Paula et al. (2011) reported that sub-lethal concentrations (0.1 ppm) of Imidacloprid in combination with the *M. anisopliae* conidia significantly reduced survival rates in adult *Ae. aegypti.*

5.5.1 Effect of exposure time

Selection of *M. anisopliae* M34412 and the concentration (1×10^{10} conidia/ml) were based on the results obtained with bioassays on fungal efficacy. In adults, an exposure time of 4 h resulted in 50 % mortality and increasing the exposure time to 48 h resulted in 93.34% mortality (Fig. 5.5).

In a similar study, Paula et al. (2008) demonstrated that a 3.5 h exposure of *Ae. aegypti* adults to *M. anisopliae* isolate at 1×10^9 conidia/ml resulted in 50 % mortality and increasing the exposure time to 48 h resulted in 89.3 % mortality with MST of 3 d. The increased exposure time means settling of the mosquitoes on the conidial card for more time leading to more adherences of the conidia. While, Stevenson (2008) observed an increase in mortality of *An. stephensi* exposed to *M. anisopliae* beyond 6 h, Scholte et al. (2003) reported no significant difference in *An. gambiae* exposed to *M. anisopliae* for 24 h, 48 h or continuous exposure.

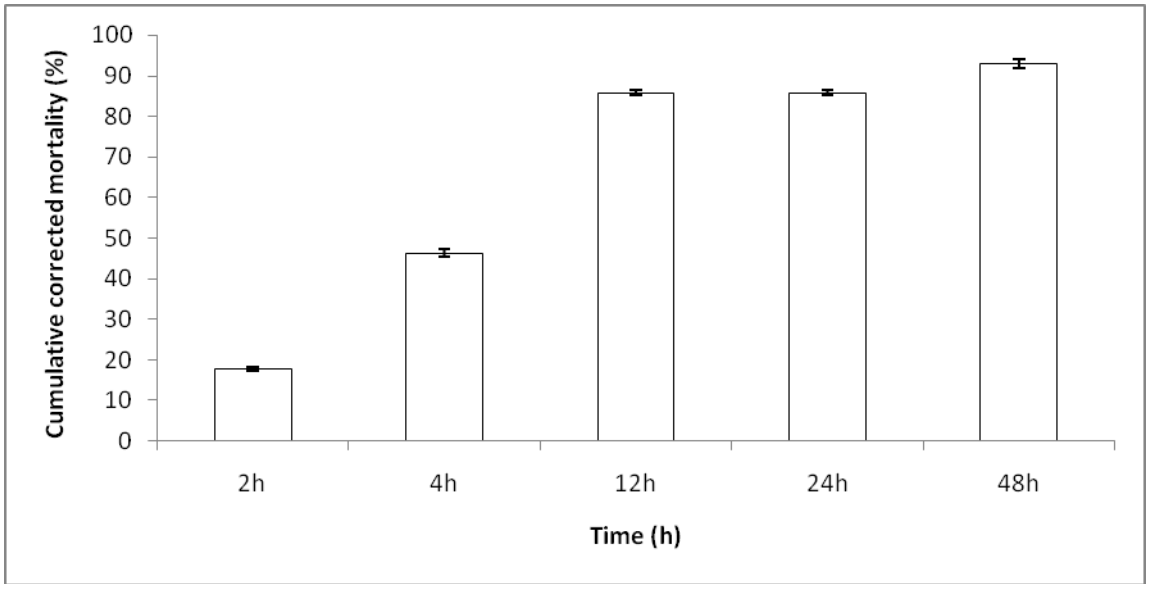


Fig. 5.5 Effect of exposure time on mortality of *Ae. aegypti* adults exposed to *M. anisopliae* M34412 at 1×10^{10} conidia/ml concentration

5.5.2 Effect of 1-4 week old *M. anisopliae* conidia on % mortality of *Ae. aegypti* adults

Mortality of mosquitoes was significantly higher during 1st week exposure which decreased gradually for exposure in 2nd, 3rd and 4th week. Scholte et al. (2005) observed a decline in infectivity of *M. anisopliae* conidia impregnated on black sheets

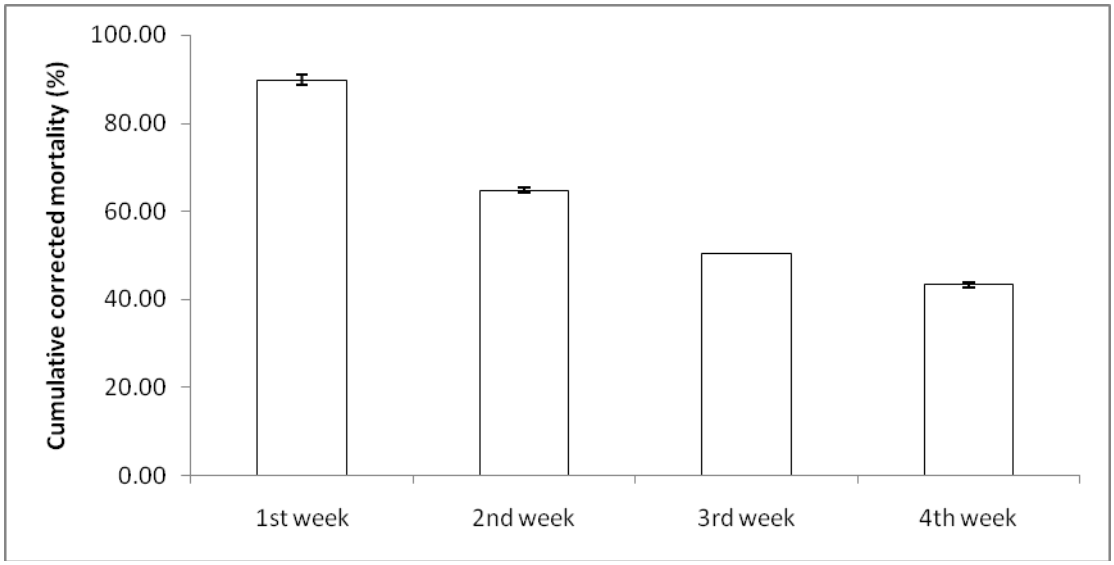


Fig. 5.6 Mortality of adults of *Ae. aegypti* exposed to same filter paper impregnated with *M. anisopliae* M34412 conidia (1×10^{10} conidia/ml) for 1-4 weeks

with a reduction in germination from 95% after day 1 to 63% after 3 weeks (Fig. 5.6).

5.6 Pathogenesis of *M. anisopliae* in *Ae. aegypti*

(i)

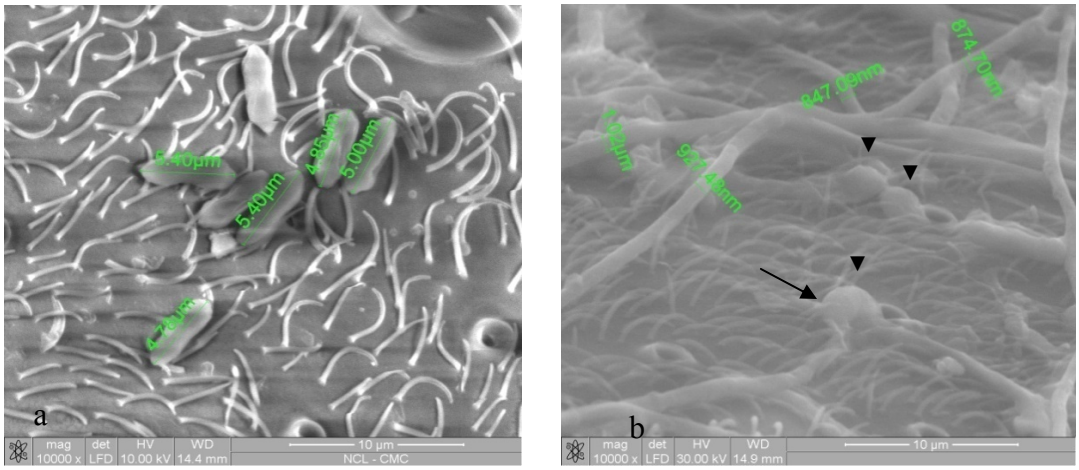


Fig. 5.7 (i) SEM micrographs showing the infection process of *M. anisopliae* M34412 in adult of *Ae. aegypti*; (a) adhesion of conidia to the host cuticle (b) Formation of appressoria (marked as '▼') leading to subsequent penetration of the cuticle (shown by arrow)

(ii)

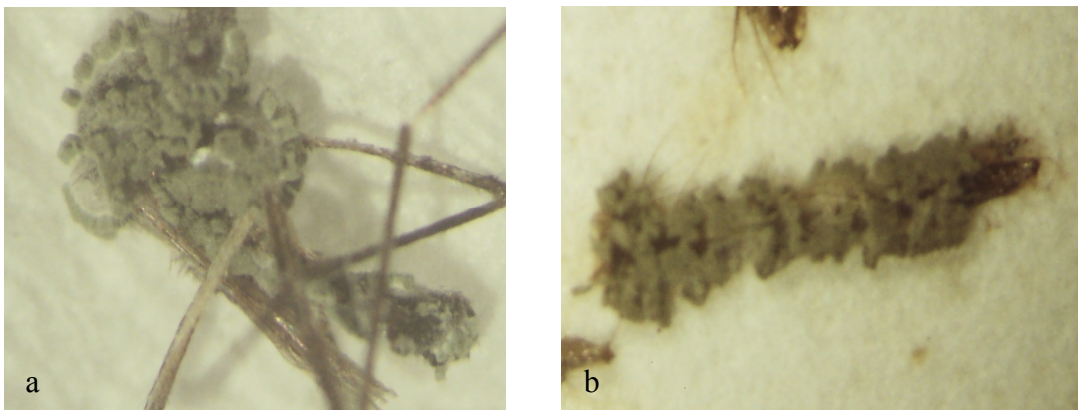


Fig. 5.7 (ii) Light microscopy images of conidiogenesis in a) adult and (b) larvae 96 h post-inoculation.

The conidia adhered to the adult mosquito surface between setae (Fig. 5.7 i (a)). After 24 h, the infection proceeded with germination of conidia and formation of globular appressoria and penetration of the host surface (Fig. 5.7 i (b)). Subsequently, the hyphae entered the body cavity, proliferated and resulted in death. Extensive

hyphal growth over the cuticle surface was observed after 96 h, in case of both adults and larvae (Fig.5.7 ii).

The present study documents the ability of *M. anisopliae* strains M34412, M34311 and M81123 in control of *Ae. aegypti*. In coclusion, either of the *M. anisopliae* strains M34412, M34311 and M81123 could preferentially be applied in integrated control programs in order to obtain maximum reduction of both larval and adult mosquito populations.

Summary and conclusions

M. anisopliae is one of most widely used biocontrol agents for the control of different insect pests. The present study was aimed to contribute in studies on different aspects of host-pathogen interactions between *M. anisopliae* and *H. armigera* which may facilitate the development of better biocontrol strategies for the control of particularly *H. armigera* and different insect pests in general. The results of the study are summarized as follows:

Sixty three *Metarhizium* isolates obtained from soil from different crop field (53) and insect hosts (10) samples were screened for *H. armigera* control. All the sixty three isolates of *Metarhizium* were pathogenic to *H. armigera*. However, the percent mortality varied from 28-97% for different *Metarhizium* isolates. The most virulent isolate M34412 showed 97.33% mortality and was obtained from soil sample of a custard apple field. Based on percent mortality, 10 isolates showing >90% mortality were selected for the determination of LT_{50}. Subsequently, based on LT_{50} (3.3 d to 6.8 d) of the 10 isolates towards *H. armigera*, five isolates M34311, M34412, M81123, M91427 and M91629 were used in LC_{50} determination (1.4×10^3 - 5.7×10^3 conidia/ml). The results of percent mortality, LT_{50} and LC_{50} values indicated that *Metarhizium* M34412, M34311 and M81123 isolates were best among all. The three *Metarhizium* isolates M34412, M34311 and M81123 were identified up to species level as strains of *M. anisopliae* by ITS sequencing and were further studied for production of conidia on a solid substrate, viability, settling time, CDE production and field application. Based on higher conidia production (67 g/kg of rice), faster sedimentation time (ST_{50}) 2.3 h in 0.1% (w/v) Tween-80, highest extracellular production of chitinase, protease and lipase (CDE complex) activities, *M. anisopliae* M34412 was selected for studies related to host-pathogen interactions and correlation to virulence. The percent efficacies with the three *Metarhizium* strains were comparable to the chemical insecticide, endosulfan, during field performance study against *H. armigera* in a chickpea crop. All three *M. anisopliae* strains, M34412, M34311 and M81123 were found to be virulent to the target pest under field conditions and possess traits necessary for being a successful mycoinsecticide.

The pathogenesis of *M. anisopliae* in *H. armigera* was studied by scanning electron and light microscopy of the different phases of fungal infection. *M. anisopliae* conidia were capable of attachment anywhere on the epicuticle surface,

although preferred attachment sites were insect setae, hair socket joints, legs and head. Conidial germination started after 12 h of inoculation and the globular appressoria of *M. anisopliae* were formed after 24 h on the larval cuticle of *H. armigera*. Direct penetration of the insect cuticle by germ tubes was also observed after 24 h, without any detectable appressoria differentiation. Thereafter, penetration was observed between 24 to 48 h post infections. Around 48 h, hyphal bodies and blastospores were observed in the haemolymph. Extensive tissue invasion and degradation over the next 24 h led to the death of the hosts within 72-96 h post inoculation. After death of the host larvae, hyphae re-emerged from the insect cadaver between 72 and 120 h. Mycelial extrusion from the cadavers between 72 and 96 h was mainly in the intersegmental areas and later, in areas with stronger cuticle, inducing complete cuticle degradation. Subsequently, around 120 h, the whole body of the larvae was covered with a white mycelial layer. Conidiogenesis was observed on the insect cadaver between 120 and 144 h after inoculation.

Previously it has been shown that repeated *in vitro* sub-culturing of *M. anisopliae* M34412 results in decline in virulence which may be attributed to the decline in hydrolytic enzyme activities. Change in the surface properties of the conidia may be another important contributor for the decreased virulence. Therefore, surface characteristics of 1st and 40th subculture conidia of *M. anisopliae* M34412 were studied. *M. anisopliae* 1st subculture conidia were found to be more virulent with LT_{50} 3.3 d as compared to 40th subculture conidia with LT_{50} 5.6 d. The germination efficiency of *M. anisopliae* conidia was found to be unaffected by repeated *in vitro* subculturing (>95% for 1st and 40th sub-culture). However, the appressorium formation was significantly reduced (~20% lower for 40th subculture). Chitinase, chitosanase, chitin deacetylase and protease activities for *M. anisopliae* from 40th subculture conidia were lower by 15-27% as compared to the activities of *M. anisopliae* from 1st subculture. The adhesion of 1st subculture conidia ($2.16\pm0.33\times10^{6}$ conidia/larvae) to the insect cuticle was found to be higher than the 40th subculture conidia ($1.90\pm0.28\times10^{6}$ conidia/larva). As no difference was observed in germination efficiency, the difference in adhesion of conidia along with more appressoria formation may be responsible for higher virulence of 1st subculture conidia than 40th subculture. The *M. anisopliae* conidia from 1st subculture settled faster (ST_{50} 2.30 h) as compared to that of the 40th subculture (ST_{50} 2.70 h) indicating that the surface of

conidia from 1st subculture may be more rough and hydrophobic. Surfaces of conidia from both the subcultures were hydrophobic, however, repeated subcuturing may have resulted in the marginal reduction in hydrophobicity index as observed for 40th subculture. Contact angle measurements confirmed that the surface of conidia from 1st subculture was more hydrophobic than 40th subculture. AFM revealed that the surface of *M. anisopliae* 1st subculture conidia was rough, which was due to presence of rodlet layers as visualized by SEM and TEM. Protein extracted from conidia was identified as hydrophobin by MALDI-TOF and LC-MS. Collectively, the study clearly showed that repeated sub-culturing causes attenuation of virulence by altering several factors responsible for *M. anisopliae* pathogenicity, more specifically CDE activities, appressorium formation and surface properties of the conidia. The data suggests that in addition to the surface properties of fungal propagules, other factors such as the properties of insect cuticle may also be playing an important role in fungus-insect interaction.

Hydrocarbons in insects consists of vast numbers of polar and non-polar aliphatic compounds, which are mainly responsible for the water balance in these insects but may also be used as carbon source for conidia germination and growth of entomopathogenic fungi. GC-MS analysis of total hydrocarbons of 3rd instar larvae of *H. armigera* over a period of 120 h following exposure to conidia of *M. anisopliae* revealed that hydrocarbon mixtures do not differ qualitatively at different periods of observation but are quantitatively distinct from one another. Over 60 major hydrocarbon components were characterised. Within the *n*-alkane portion of the hydrocarbon profile in this species, odd-chain *n*-alkanes were quantitavely present in higher amounts, the predominant one being C_{27} (heptacosane). Gradual reduction in the amounts of hydrocarbons over a period of 120 h was observed for larvae of *H. armigera* when exposed to conidia of *M. anisopliae.* Total hydrocarbons decreased from 100% at 0 h to 21.8% at the end of 120 h, which was largely due to the reduction in the amount of *n*-alkanes (from 100% at 0 h to 12.04% at the end of 120 h) thereby suggesting the utilization of *n*-alkanes as a carbon source for growth by *M. anisopliae*.

Production and surface properties of another infective propagule, blastospores were also studied. Highest production of blastospores (1.2×10^8 blastospores/ml) was obtained in Admek medium (containing Corn steep liquor) pH 5 with 10% (v/v) mycelia inoculum concentrations after 96 h. It was observed that more than 90% of

blastospores germinated after 6 h incubation while *M. anisopliae* conidia required 16 h incubation to achieve more than 90% germination. The slower rate of sedimentation indicated smooth and hydrophilic nature of blastospores surface as compared to that of conidia. Furthermore, HI (0.3) obtained by MATH assay and contact angle (50º) data confirmed the hydrophilic nature of blastospores surface. The bundles of rodlet layers were not observed in AFM, indicating the absence of hydrophobins on the surface of blastospores. Though the surface properties of conidia and blastospores were found to be significantly different, no difference was seen in their infectivity. The mortality of *H. armigera* was same with blastospores and conidia. However, the LT_{50} was found to be lower for blastospores.

Studies were conducted with *M. anisopliae* to exploit their potential for controlling insect pests of different order. *M. anisopliae* were more effective against different populations within *H. armigera*, *S. litura* and *Ae. aegypti* while less susceptible to *M. hirsutus*. The waxy coat of *M. hirsutus* may have affected the adhesion of conidia, essential to initiate infection resulting in less mortality in these insect species. The biocontrol potential of the three *M. anisopliae viz*., M34412, M34311 and M81123 was further evaluated against larvae and adult females of *Ae. aegypti*. Among the 3 *M. anisopliae* strains, M34412 was most effective and virulent particularly to the larvae of *Ae. aegypti*. In the presence of 1×10^7 conidia/ml, 3rd instar larvae of *Ae. aegypti* were susceptible to all the 3 strains of *M. anisopliae* with >95% mortality at the end of 7 d of exposure. At the lowest concentration of 1×10^3 conidia/ml, percent mortality ranged between 10-32% for all the 3 strains. In bioassays with larvae, values of LC_{50} varied from 5.92×10^3 conidia/ml for M34412, 3.49×10^4 conidia/ml for M34311 and 5.12×10^5 conidia/ml for M81123. The LT_{50} varied from 1.75 d to 3.54 d for M34412, 2.39 d to 4.46 d for M34311 and 2.66 d to 5.11 d for M81123 at the concentrations evaluated, i.e., 1×10^7 to 1×10^3 conidia/ml. A significant decrease in percent mortality of *Ae. aegypti* larvae (~20%) was observed with 40th subculture conidia as compared to 1st subculture at 1×10^3 conidia/ml concentration which may be attributed to decrease in the levels of enzyme activities and concomitant decrease in virulence during *in vitro* repeated transfers. Combination of the conidia with *M. verrucaria* CDE increased the larval mortality at lower conidial concentrations. These results suggest that *M. anisopliae* M34412 in combination with

CDE could be promising biological control agents for use against larvae of *Ae. aegypti*.

All the three *M. anisopliae* isolates exhibited significant mortality of adult *Ae. aegypti* after 8 d exposure. At highest concentration (1×10^{10} conidia/ml) tested, percent mortality values were 93.34%, 86.7% and 83.4% in adults exposed to M34412, M34311 and M81123 strains, respectively. The LT_{50} varied between 3.36 d to 5.40 d for M34412, 3.74 d to 5.45 d for M34311 and 3.98 d to 5.76 d for M81123 at the concentrations evaluated, i.e., 1×10^{10} to 1×10^{7} conidia/ml. LC_{50} values were 6.92×10^{8} conidia/ml for M34412, 5.03×10^{9} conidia/ml for M34311 and 8.22×10^{9} conidia/ml for M81123. The most promising strains, *M. anisopliae* M34412, based on virulence and stability was used for lethal exposure time determinations. An exposure time of only 4 h was necessary to cause 50% mortality.

In conclusion, present investigations led to the isolation of a highly virulent entomopathogenic fungal strain i.e. *M. anisopliae* M34412, better understanding of the pathogenesis of *M. anisopliae* and its interactions with the host insect. The study contributed significantly to the technology transfer and commercialization (currently in final phase) of *M. anisopliae* M34412 and in near future it will be available in the market as a mycoinsecticide.

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List of Publications

Research papers

Kulkarni, S.; Ghormade, V.; Kulkarni, G.; **Kapoor, M.,** Chavan, S.; Rajendran, A., Patil, S.; Shouche, Y. and Deshpande, M. V. (2008) Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. Biocontrol Science and Technology. 18: 809-828.

Kapoor, M.; Pawar, P. V.; Joseph, M.; Sen, A. and Deshpande M. V., Evaluation of biocontrol potential of *Metarhizium anisopliae* strains against larvae and adults of *Aedes aegypti* (communicated to Journal of Biological control)

Patent:

Deshpande, M.V.; Sen, A.; **Kapoor, M.** and Joshi, S. M. *Metarhizium anisopliae* strain M34412, M34311 and M81123 act as biocontrol agent against larvae and adults of *Aedes aegypti*, Ref no. INV-2012-18, NCL-28/20/12, DELL No. 1364DEL2012

Paper in symposia/Conferences/Meetings

1. **Kapoor, M.** and Deshpande, M. V. Surface characterization of entomopathogenic fungus *Metarhizium anisopliae* infective propagules. Research Scholar Meet (RSM) NCL, Pune, 27-28, February, 2012.

2. **Kapoor, M.**, Pawar, P. V., Joseph, M., Sen, A. and Deshpande, M. V. Susceptibility of larvae and adult of *Aedes aegypti* to *Metarhizium anisopliae.* Entomology Society of Amereca, Reno, USA, 13-19th November, 2011.

3. **Kapoor, M.**, Pawar, P. V., Sen, A. and Deshpande, M. V. *Metarhizium anisopliae*: Host range for its application fields in Biopesticide: Emerging Trends 3rd Biopesticide Conference held at CCS Haryana Agriculture University, Hisar, 20-22 Oct, 2010.

4. **Kapoor, M.**, Ghormade, V., Sen, A, Bhat, S. and Deshpande, M.V. Host-pathogen interaction in *Metarhizium anisopliae* and its insect host *Helicoverpa armigera* (Hübner) in 50th Association of Microbiologist Annual Conference at National Chemical Laboratory, Pune 15-18 Dec, 2009.

5. **Kapoor, M.**; Kadam, M.; Ghormade, V.; Chavan, S.; Kulkarni, S.; Rajendran, A. and Deshpande, M. V. Formation and regeneration of protoplasts from mycelial and blastospores of *Metarhizium anisopliae* at 48th Association of Microbiologist of India Annual Conference at Department of Biotechnology, Indian Institute of Technology, Chennai, 18-21 Dec, 2007.

6. **Kapoor, M.;** Kondawar, V.; Chavan, S.; Kulkarni, S. and Deshpande, M.V. Morphological transitions in entomopathogenic fungi *Metarhizium, Nomuraea* and *Beauveria*. Poster presented at Biology of Yeast and Filamentous Fungi at National Chemical Laboratory, Pune, India, Feb 15-17, 2007.