TABLET FORMULATION OF ENTOMOPATHOGENIC FUNGUS AND ITS BIOEFFICACY IN MOSQUITO CONTROL

by

NEEMA DILEEP

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Neema Dileep

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LIST OF ABBREVATIONS

@	At the rate of
^{0}C	Degree Celsius
CD	Critical Difference
CRD	Completely Randomised Design
cfu	colony forming units
DAS	Days After Storage
WAS	Weeks After Storage
MAS	Months After Storage
DAT	Days After Treatment
EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematode
et al.	And others
g	Gram
g^{-1}	Per gram
h	Hours
HAT	Hours After Treatment
L^{-1}	Per litre
m^{-2}	Per meter square
mg	Milligram
mL	Millilitre
mL^{-1}	Per milliliter
NS	Non Significant
sp. or spp.	Species (singular and plural)
s	Seconds
min	Minutes
viz.	Namely
CMC	Carboxy Methyl Cellulose
MCC	Microcrystelline Cellulose
PVP	Polyvinyl Pyrrolidone
AG	Acacia Gum Arabic

Introduction

INTRODUCTION

Microbes can be successfully used as a biological weapon against deadly mosquito borne diseases as they are sustainable, ecofriendly and safe compared to chemical insecticides. Entomopathogenic bacteria and fungi have high potency to control all the stages of mosquitoes and can be used as an excellent alternate to chemical pesticides. *Bacillus thuringiensis* var. *israelensis* Barjac *(Bti)* is the most common microbe employed worldwide for mosquito control (Federici *et al.,* 2003). Entomopathogenic fungi belonging to oomycota, chytridiomycota, zygomycota and deuteromycota have been reported to infect and kill the different mosquito species (Scholte *et al.,* 2004). There is a renewed interest in using these fungi for mosquito control due to the ever increasing spread of vector borne diseases and the risk imposed due to development of pesticide resistance in mosquitoes.

The state had been haunted by mosquito borne diseases like malaria, lymphatic filariasis (Feroze and Aravindan, 2001); dengue fever (Das *et al*., 2004) and chikungunya (Kannan *et al*., 2007). *Anopheles*, *Aedes* and *Culex* are the major vectors that are pathogenic to both human and other domesticated animals (Sumodan, 2014). They breed in stagnant water bodies and therefore, treating these water bodies with chemicals imposes risk of drinking water pollution as well as depletion of biodiversity of the aquatic flora and fauna.

Larval stage is the most perfect stage for biocontrol in mosquitoes. Biocontrol approaches to manage these immature stages are essentially needed to minimise the troublesome impact of synthetic insecticides on environment and mankind. Tapping the potential of entomopathogens for managing mosquito larvae would be a safer method for their management. Furthermore, entomopathogens pose minimal risk to the persons engaged in spraying. Apart from this, they do not act upon the natural enemies or other organisms in water bodies and thereby prevent resurgence and resistance development (Zimmerman, 2007).

Entomopathogenic fungi (EPF) have been investigated for their potential use as biopesticides in pest control. Fungi such as *Metarhizium anisopliae* (Metsch.) Sorokin, *Beauveria bassiana* (Bals.) Vuillemin and *Lecanicillium lecanii* (Zimmerman) Zare and Gams can be effectively employed in mosquito control as

they are reported to cause infection in all the three mosquito species (Vivekanandhan *et al.*, 2020a). A major impediment that limits the adoption of EPF in mosquito control is the lack of stable formulations. More often, it is not the lack of bioformulations, but the lack of proper delivery system that leave them behind in the global pesticide market. Formulation improvement is therefore an important strategy to enhance the wide spread usage of biopesticides.

Among the various formulations used in mosquito control, EPF formulations have been limited to either oil or wettable powder (Daoust *et al.*, 1983). These conventional approaches produce a lot of dust or may contain large amount of solvent. Powder formulations absorb moisture, leading to contamination and subsequent loss of viability of the active ingredient, whereas oil formulations suffer poor spraying characteristics. The drawback of conventional formulations should not be an obstacle for widespread use of promising biopesticides. Smart formulations such as tablets, granules, capsules, gels etc. can overcome this obstacle.

Water dispersible tablet is a compressed formulation that disintegrates in water or other liquid with the release of active ingredient to produce a stable and homogenous suspension (FAO, 2010). Highly compressed nature of the tablets often leads to lesser absorbance of moisture and contamination by saprophytic fungi. They can be placed directly in mosquito breeding sites for self-dispersal of the active ingredient. This minimises the risk of dustiness and contamination and at the same time enables easy handling and reduced inoculum wastage.

Although *Bti* tablets are available in the market for mosquito control, exploitation of entomopathogenic fungi or their improved formulations are lacking. The present research work entitled "Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control" was therefore undertaken with the following objectives

- Assessment of pathogenicity of entomopathogenic fungi to the common mosquito species of Kerala.
- Determination of effective dose, lethal concentration and lethal time of the most effective fungus.

- Standardisation of carrier material to develop a tablet formulation of the fungus
- Standardisation of binding agent suitable for compressed tablets
- Determination of ideal moisture content for the formulation
- Shelf life of the tablet under ambient conditions
- Bio efficacy of the tablet in managing mosquito larvae in stagnant water

Review of Literature

2. REVIEW OF LITERATURE

Mosquitoes (Diptera : Culicidae) are a key threat for millions of people worldwide, since they act as vectors for devastating pathogens and parasites. They affect the health and well-being of human population by transmission of vector - borne diseases and the nuisance associated with mosquito bites. Even though the application of chemical insecticides in the breeding sites, is the best strategy to kill larvae of mosquitoes in water, the negative effect of chemicals on non-target organisms, environmental pollution, resistance to these chemicals in mosquitoes, along with the recent resurgence of different diseases transmitted by mosquitoes, have led to the search of microbial control methods, which are more safe and sustainable for mosquito control. The review of literature pertaining to mosquito species reported as vectors, with more emphasis to Kerala and their management strategies is presented below.

2.1 MOSQUITOES AS VECTORS OF HUMAN DISEASES

Mosquitoes transmit several devastating diseases in India. A serious outbreak of dengue hemorrhagic fever was reported in India, in 1996 which caused the death of several people (Dar *et al.*, 1999). Japanese encephalitis, a viral brain infection affected more than 50000 people across the country (Mackenzie *et al*. 2007). Chikungunya, a rarely fatal, but severely symptomatic infection, was first reported during 1963 in Kolkata (Ramachandran, 2006). Later in 2017, two major outbreaks of Zika virus were reported in Gujarat and Tamil Nadu (Bhardwaj *et al.*, 2017). The worst mosquito borne disease that was reported worldwide was malaria. According to WHO (2018), among different South East Asia Region countries, India itself accounts 41 per cent malarial deaths, indicating it as one of the most fatal disease in the country.

Kerala had been affected by these mosquito borne diseases for several years. Sumodan (2014) recorded 118 species of mosquitoes under 15 genera from Kerala, among which *Anopheles, Culex* and *Aedes* are the major genera reported as vectors.

2.1.1 *Anopheles*

About 58 species of *Anopheles* had been reported in India, of which six species are implicated as primary vectors transmitting malaria in different geographical regions. The species reported are *Anopheles culicifacies* Giles, the vector in rural areas, *Anopheles fluviatilis* James in the plains and foothills, *Anopheles minimus* Theobald breeds in streams of foothills of the northeast, *Anopheles dirus* Peyto and Harrinson in jungles of northeastern states, *Anopheles sundaicus* (Rodenwaldt) found in Andaman and Nicobar islands and breeds in brackish water, and *Anopheles stephensi* Liston, the vector species of urban malaria (Subbarao, 2019).

In Kerala, first report of *Anopheles* spp. were given by Christophers (1933) and the species were *An. culicifacies*, *An. fluviatilis* and *An. stephensi*. Further, *Anopheles sinensis* (Wiedemann) was reported by Balasubramanian and Nikhil (2013), which is also a vector of malaria.

2.1.2 *Aedes*

About 20 species of *Aedes* had been reported from various locations in India, of which *Aedes aegypti* Linnaeus is a highly anthropophilic mosquito, which breeds in man-made containers, whereas *Aedes albopictus* Skuse is another important dengue vector in rural areas (Tyagi *et al.*, 2015).

Ae. aegypti and *Ae. albopictus* were reported as transmitters of dengue fever, chikungunya, yellow fever and zika virus by Barraud (1934) from Kerala, while Balasubramanian and Nikhil (2013) reported *Ae. greenii* Theobald and *Aedes w-albus* Theobald too as vectors of aedes arbovirus.

2.1.3 *Culex*

In India, about 85 species of *Culex* have been reported, of which the common species reported to cause filariasis and Japanese encephalitis are *Culex quinquefasciatus* Say, the most common domestic species in urban, semi urban and rural areas, *Culex tritaeniorhynchus* Giles, *Culex vishnui* Theobald extremely common species that mainly breed in paddy fields, *Culex gelidus* Theobald that prefer

marshy depressions containing abundant aquatic vegetation, *Culex whitmorei* (Giles) which breeds in freshwater ground pools (Philip *et al.*, 2010).

In Kerala, *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* were reported by Giles (1901), while *Cx. vishnui* was reported by Barraud (1934). Balasubramanian and Nikhil (2013) reported *Culex sinensis* Theobald, *Culex malayi* Leicester, *Culex mimuloides* Barraud and *Culex pallidothorax* Theobald from various locations of Kerala.

2.2 MICROBES USED IN MOSQUITO CONTROL

Microbial insecticides comprises living organisms such as viruses, bacteria, fungi, protozoa, nematodes or their products or toxins. Microbes can be successfully used as a biological weapon against deadly mosquito diseases as they are sustainable, ecofriendly and safe compared to chemical insecticides. A number of microbes have been reported from larval and adult mosquitoes including bacteria, viruses, fungi, nematodes and protozoa (Lacey and Undeen, 1986).

2.2.1 Entomopathogenic Bacteria

Bacterial pathogens generally used for insect control are spore-forming and rod-shaped coming under the genus Bacillus. *Bacillus thuringiensis* Berliner, *Bacillus popillae* Dutky, *Bacillus lentimorbus* Dutky, and *Lysinibacillus (Bacillus) sphaericus* Meyer and Neide are the common entomopathogenic species. Among these, *B. thuringiensis* is the most widely and successfully used bioinsecticide in the integrated pest management programs, worldwide representing about 90 per cent of the all biological insecticides marketed across the world (Jallouli *et al.*, 2020).

The pathogenic action of this bacterium normally occurs after ingestion of spores and crystalline inclusions containing insecticidal δ-endotoxins that specifically interact with receptors in the insect midgut epithelial cells (Pigott and Ellar, 2007).

The most potent bacterial agents infecting mosquitoes comprise of *B. thuringiensis* subsp. *israelensis* Barjac *(Bti)* and *L. sphaericus.* Promdonkoy and Ellar (2003) reported that *Bti* produce key proteins, Cry toxins and Cyt toxins that interact with multiple midgut receptors leading to death of the host insect, whereas

L. sphaericus produces a single binary toxin, Bin that binds with a specific receptor α-glucosidase (Federici *et al.,* 2003).

2.2.2 Entomopathogenic Viruses

Viruses, like bacteria, must be ingested to infect insect hosts. The larvae of many insect species are vulnerable to devastating epidemics of viral diseases and they are very specific, usually acting against only a single insect genus or even a single species (Caballero and Hill, 1992). Nucleopolyhedrosis virus (NPV) and granuloviruses are the main entomopathogenic viruses used in insect pest management (Hu *et al.,* 2003) in which NPVs have great potential against many lepidopteran pests (Tanga *et al.,* 2011).

Development and use of virus-based insecticides have been limited due to the fact that unlike *B. thuringiensis*, insect viruses must be produced in live host insects (Narayana, 2003). Production is therefore both expensive and time-consuming.

The most common occluded viruses infecting mosquitoes are the baculoviruses whereas non occluded viruses are represented by the densoviruses. Mosquito densoviruses (MDV) replicate in the nuclei of mosquito cells and cause the characteristic nuclear hypertrophy (densonucleosis). White coloured cuticle with dark shiny areas of melanisation, with malformed segments and curved abdomen are the symptoms of the infection (Jousset *et al*., 2000). Baculoviruses found in mosquitoes are restricted to the NPV group. NPV infections are detected by the hypertrophied nuclei of midgut cells that appear white due to the accumulation of occlusion bodies (Becnel and White, 2007).

2.2.3 Entomopathogenic Fungi

Fungi have proved to be effective for the control of several insect and mite pests due to their unique mode of action. Unlike bacteria and viruses they can infect upon contact with the treated surface. The main route of entrance of the entomopathogenic fungi (EPF) is through integument, trachea or through wounds (Holder *et al.,* 2005).

Fungi belonging to over 100 genera contribute a key share of commercially exploited microbes (St. Leger and Wang, 2010). *Metarhizium anisopliae* (Metch.) Sorokin), *Beauveria bassiana* (Bals.) Vuillemin, *Beauveria brongniartii* (Sacc.) Petch, *Lecanicillium lecanii* (Zimm.) Zare and Gams, *Isaria fumosorosea* (Wize) Brown and Smith, *Hirsutella thompsonii* (Fisher) and *Cladosporium cladosporioides* Fresenius are those which have been developed as mycoinsecticides (Maina *et al.*, 2018).

Among the various biopesticides based on EPF in India, *B. bassiana* ranks first (17 per cent), followed by *Lecanicillium* spp. (15 per cent) and *M. anisopliae* (6 per cent) (Mishra *et al.*, 2020).

Entomopathogenic fungi can be grown in massive amounts on inexpensive artificial media, and conidia can be stored easily. Moreover, its failure to germinate until the actual exposure to a host and its resulting persistence in the environment make EPF a very promising control agent (Roberts, 1970). Among the microbial agents infecting mosquitoes, EPF are host specific and are known to cause natural infection and also epizootics (Geetha and Balaraman, 1999).

Vivekanandhan *et al*. (2018) reported that the common genera that are infective to mosquitoes are *Beauveria*, *Metarhizium*, *Leptolegnia*, *Pythium*, *Lagenidium*, *Coelomomyces*, *Conidiobolus* of which, *B. bassiana* and *M.anisopliae* has the immense potential to act as a biocontrol agent for mosquitoes

2.2.3.1 B. bassiana as a biocontrol agent for mosquitoes

Conidial suspension of *B. bassiana* @ 5×10^5 conidia mL^{-1} caused 100 per cent mortality to adults of *Culex* spp., *Aedes* spp. and *Anopheles albimanus* (Wiedemann) within five days of exposure. In small scale outdoor experiments, *B. bassiana* @ 5×10^5 conidia mL^{-1} caused 69 to 95 per cent reduction in population of larvae and pupae of *Culex pipiens* L. two weeks after treatment (Clark *et al*., 1968).

Grove and Pople (1980) reported that *B. bassiana* can cause 39 per cent mortality of *Ae. aegypti* larvae when applied as toxin beauvericin @ 10µg mL^{-1} whereas at 20µg mL^{-1}, it caused a mortality of 86 per cent after 48 h of treatment.

Geetha and Balaraman (1999) proved that *Cx. quinquefasciatus* larvae are more susceptible to *B. bassiana* than *Anopheles* sp. whereas *Aedes* sp. is resistant to the infection. Results on time-mortality responses showed that second instar larvae of both the species had low LT_{50} value (104.35 and 96.56 h) than the third instar (109.64 and 113 h) showing that the second instar is more susceptible to *B. bassiana* infection than the third instar.

B. bassiana can significantly reduce blood feeding in *Anopheles* after they acquire a fungal infection and can be used as a new mosquito control tool for reducing malarial disease transmission (Howard *et al.,* 2010). García – Munguía *et al.* (2011) reported that *B. bassiana* infected male mosquitoes, could transfer the fungus to uninfected females by mating behaviour which exerted a negative effect on egg production. Females exposed to the virulent strain Bb-CBG2 had a mean fecundity of 2.05 (± 1.02) eggs per female, which was 95 per cent lower than the mean fecundity of healthy females in control group which was 42.56 (± 6.90).

Paula *et al*. (2018) suggested PET trap method of using black cloths which are attractive to mosquitoes. Impregnating these cloths with conidia of *B. bassiana* for trapping mosquitoes effectively controls adult *Ae. aegypti* by 68 per cent.

2.2.3.2 M. anisopliae as a biocontrol agent for mosquitoes

Ferron (1981) reported that *M. anisopliae* toxin "destruxin" kill the host by inciting degeneration of host tissues due to structural integrity of membranes and dehydration of cells by fluid loss. *M. anisopliae* reduced the blood feeding and fecundity of *Anopheles gambiae* Giles and can be used as a potential biocontrol agent (Scholte *et al.,* 2004).

Farenhorst *et al.* (2008) demonstrated the potential of *M. anisopliae* against mosquitoes by using clay water pots as resting sites of *Anopheles* spp. adults. They reported that application of 10^{10} conidia m^{-2} infected 92 per cent of *An. gambiae* and *Anopheles funestus* Giles.

M. anisopliae isolated from dead adult mosquitoes obtained from the surrounding environment when administered to larvae of *Ae. albopictus*, exhibited

high larvicidal action with LC_{50} value 1.09 X 10^5, LC_{90} 1.90 X 10^{13}, and LT_{50} 45.41 h (Bilal *et al*., 2012).

Benserradj and Mihoubi (2014) tested the virulence of *M. anisopliae* using five different spore concentrations of 10^9, 10^8, 10^7, 10^6 and 10^5 conidia mL^{-1} against 4th instar larvae of *Cx. pipiens* and found that the larval mortality increases with increase in concentration. Secondary metabolites of *M. anisopliae* when applied on *Ae. aegypti*, *An. stephensi* and *Cx. quiquefasciatus*, resulted in 85, 97 and 89 per cent mortality respectively. The corresponding LC_{50} values were 59.83µg mL^{-1}, 50.16 µg mL^{-1} and 51.5 µg mL^{-1} (Vivekanandhan *et al.*, 2020b).

Lwetoijera *et al.* (2010) evaluated the efficacy of *M. anisopliae* baits prepared by impregnating fungal spores on black cotton cloths, against the malaria mosquito *Anopheles arabiensis* Giles. Bait stations which were rural regions away from human habitations were visited by mosquitoes and they found that 95 per cent of mosquitoes that visited the stations died within 14 days. They also used a yeast-based bait to generate CO_2, for attracting the mosquitoes.

2.2.4 Other Microbes Used in Mosquito Management

2.2.4.1 Nematodes

Entomopathogenic nematodes belonging to the families, Heterorhabditidae and Steinernematidae have been used against insects and arthropods of medical and veterinary significance, including cat flea, flies, mosquito larvae, black flies, body louse, ticks and head louse (Begley, 1990). The infective juveniles of the nematodes, *Heterorhabditis* sp. and *Steinernema* sp. have been used against insect pests, which carry symbiotic bacteria, *Photorhabdus* and *Xenorhabdus*, respectively. These bacteria multiply in the insect body and the host insect dies within two to three days (Ferreira and Malan, 2014).

The mermithid nematode *Romanomermis culicivorax* Walker was studied extensively in North America and was found effective to *An. albimanus* and *Cx. nigripalpus* in several natural habitats of Oaxaca, Mexico. Inoculation of 500 to 1000 preparasites m^{-2} resulted in 74 to 88 per cent infection in *Anopheles* and and 77

to 97 per cent infection in *Culex* (Perez-Pacheco *et al.,* 2009). Later, another species of mermithid, *Romanomermis iyengari* Welch, which was originally found in India, has been investigated for use in Asia and Central America, for controlling mosquitoes. Monthly application of 3500 second stage juveniles m^{-2} was enough to effectively control larval *An. gambiae* in wetlands (Abagli *et al.,* 2019).

2.2.4.2 Protozoans

Entomopathogenic protozoans are host specific, slow acting and produce chronic infections to a wide range of insect hosts (Solter and Becnel, 2007). Species in the genera *Nosema* and *Vairimorpha* offer immense potential for use as insecticides. Although protozoans play a significant role in the natural limitation of insect populations, few appear to be suited for development as insecticides (Sarwar *et al.,* 2021).

Protozoans infecting mosquitoes are mainly represented by Ciliophora. Among Ciliophora, *Lambornella clarki* (Corliss and Coats), *L. stegomyiae* Walker, *Tetrahymena pyriformis* (Ehrenberg) and *Chilodonella uncinata* (Ehrenberg) are the known endoparasites of mosquito larvae (Das, 2003).

2.4 BIOFORMULATIONS FOR PEST MANAGEMENT

Formulation is one of the most important aspects that determines the feasibility of a microbial agent. It is a mixture of active ingredient with inert or inactive carrier materials. Burges and Jones (1998) stated that bioformulation comprises of the aids to preserve organisms and also the mechanisms to deliver them to their targets. Development of a good formulation is critical for commercial biopesticides to be used successfully.

2.4.1 Types of Bioformulations

Biopesticides are usually formulated as dry or liquid formulations. Dry formulations are prepared mostly in solid carriers such as talc, peat, lignite, clay, and other similar materials whereas liquid formulations are prepared in non-aqueous liquid intended for dilution before use.

Anderson and Roberts (1983) defined the essential constituents of a liquid formulation, as active ingredient (10 – 40%), carrier liquid (35 – 65%), suspensor ingredient (1 – 3%), dispersant (1- 5%) and surfactant (3 – 8%). Seaman (1990) defined various constituents in a dry formulation as active ingredient (50 – 80%), carrier (15- 45%), dispersant (1 -10%) and surfactant (3- 5%). Burges and Jones (1998) stated that dry bioformulation should contain 5–20% of organism, usually less than 15% in granules, pellets, capsules and briquettes.

2.4.1.1 Conventional formulations

The basic conventional formulations are dust, wettable powder, granule and liquid formulations or oil formulations.

2.4.1.1.1 Dust

Dusts are made by saturating an active ingredient with a finely ground solid mineral powder such as talc or clay with particle size ranging from 50 to 100 microns. *B. bassiana* formulated in kaolin showed 98.75 per cent mortality after seven days of treatment, followed by talc and tapioca flour formulation. Whenever the insects moved in the rice grains, waxy layer of the integument was abraded and removed, allowing the conidia to stick on and penetrate through the exoskeleton, thus hastening the infection (Samodra and Ibrahim, 2006).

Oi *et al.* (1994) injected conidial powder formulation of *B. bassiana* into ant mounds, resulting in 52 to 60 per cent reduction in the population of the imported red fire ant, *Solenopsis invicta* Buren. Conidia dust formulation of 25% *M. anisospliae* isolate IRAN 437C, with wheat flour as carrier when applied on brown banded cockroach, *Supella longipalpa* F. a vector of many human pathogens, caused high mortality in nymphs and adults (100 and 92.5 per cent mortality) at seven days after exposure under room conditions (Sharififard *et al.,* 2013). Vinayaka *et al*. (2018) proved the efficacy of *M. anisopliae* dust at 10^8spores mL^{-1} in killing arecanut white grub *Leucopholis lepidophora* Blanchard that caused 83.33 per cent death.

2.4.1.1.2 Wettable powders

Wettable Powders (WP) are fine dry powders that are applied after being suspended in water and made by combining an active ingredient with a surfactant, wetting and dispersing agents, inert fillers and then grinding the mixture to the desired particle size (about five microns).

Olson and Oetting (1999) found that *B. bassiana* WP formulation @ 2 x 10^{10} cfu g-^{1} reduced the population of the silver leaf whitefly, *Bemisia argentifolii* Bellows and Perring, with significant reduction in adult emergence. Nagaraja (2005) prepared bentonite and glucose based (7:1) WP formulation of *M. rileyi* and reported 87 and 79 per cent cumulative mortality of *Helicoverpa armigera* Hubner and *Spodoptera litura* F. under laboratory conditions. Ihsan and Ibrahim (2007) reported that *B. bassiana* WP formulation @1 x 10^{10} conidia mL^{-1} resulted in significant recovery of chilli shoots in the field, seven days after treatment with no infestation of mite *Polyphagotarsonemus latus* (Banks). The shoot recovery rate was 93.33 per cent, which was equally effective to that of Amitraz (96.67 per cent). WP formulations of 10% *B. bassiana* and *M. anisopliae* @ 1 x 10^{8} spores mL^{-1} when applied against the spiny boll worm *Earias insulana* Boisduval exhibited a considerable reduction in number of infested bolls after 14 days of treatment under field conditions (Loufty and Mustafa, 2021).

2.4.1.1.3 Granules

Jaronski and Jackson (2008) investigated the effect of soil application of microsclerotial granules (MS) of *M. anisopliae* grown in medium with a high C: N ratio (50:1) against the sugar beet root maggot, *Tetanops myopaeformis* (Roder), and found that it can be used as a viable formulation for the pest and was highly effective even in low moisture levels of soil. Granular formulations of *B. bassiana* and *M. anisopliae* applied to the upper layer of potting soil of marigold plants to target the late larval and pupal stages of western flower thrips, *Franklinella occidentalis* Pergande, was found to reduce the damage till eight weeks and thereafter the population was suppressed and foliar damage was minimized (Skinner *et al.,* 2012).

Kim *et al.* (2014) reported that the application of millet based *B. bassiana* granules @ 1 x 10^{8} conidia g^{-1} to soil caused 83.3 per cent mortality of rice water

weevil, *Lissorhoptrus oryzophilus* Kuschel, two days after treatment, under field conditions.

Population of *F. occidentalis* on eggplants grown in soil impregnated with *B. bassiana* granules was 70 per cent lower than those on untreated soil after eight weeks of treatment, in green house conditions. They also observed that the survival and growth of *B. bassiana* on granules was more when soil moisture content was 20 per cent (Zhang *et al.,* 2019).

2.3.1.1.4 Liquid / Oil formulations

Higher efficacy of oil based formulations might be due to prevention of the desiccation of the conidia which helps in longer survival period and better penetration of peg infection into the integument (Burges and Jones, 1998). Oil could coat the dry, dusty type of conidia allowing them to suspend easily in oil and spread rapidly over the surface of leaves which helps better contact of conidia with insect cuticle.

Bateman *et al*. (1992) opined that application of *M. flavoviridae* formulated in cotton seed oil against desert locust *Schistocerca gregaria* Forsskal exhibited superior performance than a water-based suspension, and that it killed the pest faster in dry conditions with less than 35% relative humidity. When compared to sunflower oil, Verhaar *et al*. (1999) found groundnut oil to be a better carrier for *L. lecani*, with high germination. Alves *et al.* (2002) observed the effects of oils like refined paraffin oil and vegetable oil (peanut oil, sunflower oil, and soyabean oil) on conidial viability of *Metarhizium* and found that the formulation based on groundnut oil maintained over 90 per cent viability even after 40 weeks of storage. According to Banu and Gopalakrishnan (2012), sunflower oil was found to be superior in retaining the viability of *L. lecanii* spores when compared to talc-based formulations and the oil formulations were highly effective (80 per cent mortality) against the mealy bug *Paracoccus marginatus* Williams and Granara, infesting cotton.

Corn oil-based formulation of *B. bassiana* was effective against chilli mite *P. latus*, with a reduction of 57.51 per cent population after two rounds of spraying (Sangeetha, 2013). Wraight *et al.* (2016) examined the effectiveness of paraffin Oil Dispersion (OD) and clay-based WP formulations of *B. bassiana* against the melon

aphid, *Aphis gossypii* (Glover), and concluded that OD is superior to WP as the mortality of the test insect was increased by 27 per cent. Chitin enriched oil formulation of *L. lecanii*, both the groundnut oil and sunflower oil was equally effective as carriers with a viability of 2.27 x 10^6 and 2.20 x 10^6 spores mL^{-1} under room temperature after 3 months of storage (Nithya and Reji, 2017).

2.4.1.2 Novel formulations

Novel formulations are intended to reduce the bulkiness in storage, contamination and subsequent loss of viability of active ingredient. They can be delivered precisely to the target and would enhance the field performance of many potential entomopathogens. Capsules, microcapsules, gels, pellets and tablets are some of the novel formulations.

2.4.1.2.1 Capsules

Capsule is a stable formulation wherein the active ingredient is encapsulated within coatings and thus protected from extreme environmental conditions and its residual stability is increased by slow or controlled release (Burges and Jones, 1998).

Hiltpold *et al.* (2012) established a technique for producing capsules, in which the nematode *Heterorhabditis bacteriophora* Poinar was encapsulated in a polysaccharide shell derived from the algae *Laminaria* sp. These capsules when buried in the rhizosphere of maize were found to be effective in controlling the western corn rootworm, *Diabrotica virgifera* LeConte. Kim *et al.* (2015) prepared EPN capsules of *H. bacteriophora* and suggested that more evenly produced capsule shell structure will result in greater capsule hardness, with improved retention of EPN retainment. Retention and hardness of alginate capsules were considerably improved when they were treated with calcium.

Capsules of entomopathogenic fungi were first formulated by Remya and Reji (2020). Based on disintegration studies in plants and soil, in relation to moisture, temperature and relative humidity, they standardised the coating material and carrier material for capsule formulations of *B. bassiana* and *M.anisopliae*. The study concluded that gelatin capsules with talc or chitosan as the carrier material is ideal for

the preparation of biocapsules of EPF intended for immediate release while, Hydroxy Propyl Methyl Cellulose (HPMC) was the coating material for slow release. Chitosan was observed as the better carrier for controlled release of inoculum.

Gola *et al.* (2019) formulated *B. bassiana* in prefabricated gelatin capsules in rice flour to study the ability of the fungus in multi metal removal from synthetic waste water and observed 93 per cent of multimetal removal ability.

2.4.1.2.2 Microcapsules

Microcapsules based on microbes are prepared by microencapsulation technique. The goal of encapsulation is to provide a microenvironment to the microbes, within a capsule, allowing them to survive during processing and storage (Weinbreck *et al.,* 2010). Microbial cells form the core of the formulation that in turn is coated with a polymeric material which acts as the shell (Schoebitz *et al.,* 2013).

Low-temperature spray drying was used to microencapsulate the conidia of *B. bassiana* using a matrix composed of 10% dextrin, 10% skimmed milk, and 5% PVP K90 as the coating material. The conidia exhibited 80 per cent viability, even after storage for six months at 4^0C (Liu and Liu, 2009).

Pathogenicity of bovine gelatin microencapsulated spores of *B. bassiana* and *M. anisopliae* @ 1 x 10^8 spores mL^{-1} reduced the spore viability from 86.6 to 33.3 per cent for *B. bassiana* and 86.6 to 56.6per cent for *M. anisopliae* due to the dehydration of fungal spores (Jimenez *et al.,* 2015). Microencapsulation of *B. bassiana* conidia with sodium humate showed good viability at the end of six months of storage at room temperature (Qureshi *et al.,* 2015). These formulations were effective to *H. armigera* larvae causing 93 per cent mortality within five days of treatment, under laboratory conditions. Qui *et al.* (2019) developed microcapsules of *M. anisopliae* through a new microencapsulation method, complex coacervation, based on gelatin and gum arabic. It provided protection against UV and exhibited longer shelf life when compared to non-encapsulated conidia. It was effective to imported red fire ant *S. invicta* causing 90 per cent mortality.

2.4.1.2.3 Gel

Gels have intermediate properties of solids and liquids and are suitable for solid formulations (Perrin, 2000).

Chang and Gehret (1991) encapsulated *Steinernema carpocapsae* Weiser in a matrix of macrogels, a partially hydrogenated vegetable oil paste containing mono and diglycerides, which significantly prolonged the viability. Navon *et al.* (2002) developed *S. carpocapse* gel formulations @ 1000 infective juveniles g^{-1} and observed complete mortality when these were treated against fourth instar of *Spodoptera littoralis* Boisduval and *H. armigera.*

Remya (2018) found that the chitosan-based gel of *B. bassiana* and *M. anisopliae* was superior in terms of spore germination rate and shelf life and had less contamination. In a pot culture study, she found that pit treatment with chitosan based gel of *M. anisopliae* in the planting pit @ 10 g $plant^{-1}$ resulted in a 61.11 per cent reduction in *Cosmopolites sordidus* grubs, when applied as prophylactic measure, while curative application resulted in only 36.11 per cent reduction in population.

2.4.1.2.4 Pellets

Pellets are solid masses of more than 10 mm^3 size, manufactured by mixing as a slurry or thick liquid which is then extruded under pressure like a long sausage and cut into a uniform shape (Burges and Jones, 1998).

Potential of alginate pellets of *B. bassiana* formulated with or without wheat bran was explored by Knudsen *et al.* (1990) in controlling cereal aphid, *Schizaphis graminum* (Rondani). It was found to cause 48 per cent mortality with profuse fungal sporulation. White (1995) suggested that poly ethylene glycol treated *B. bassiana* alginate pellets sporulated sooner than the untreated ones and that the former caused greater mortality more quickly in imported red fire ant *S. invicta* and suggested that ingredients in alginate formulation can be manipulated to increase the rate of mortality.

Hidalgo *et al.* (1997) observed that 24 h of direct contact of stored grain pests *Sitophilus zeamais* Motsch with the rapeseed oil pellet formulation containing 10^{10} conidia g^{-1} gave 100 per cent mortality after seven days. The formulation also

maintained a conidial viability of 84.7 per cent, 45 days after storage. Bextine and Thornvilson (2002) formulated *B. bassiana* alginate pellets coated with peanut oil and observed 80 per cent reduction in the activity of *S. invicta.*

2.4.1.2.5 Tablets

Tablets are compressed mass of active ingredient along with other additives and have usually a circular shape.

de Medeiros *et al*. (2005) developed tablet formulation of *L. sphaericus* and found it to be effective as a larvicide against *Cx. quinquefasciatus* resulting in 100 per cent mortality. They opined that the tablet is a are better formulation with the advantages of low dosage, uniformity in application, stability in storage, easiness in transportation and field efficacy. Armengol *et al.* (2006) demonstrated prolonged control of *Ae. aegypti* in containers kept in full sunlight added with tablet formulation of *Bti*. Kala *et al*. (2019) suggested that neem oil water dispersible tablet with concentration of 40 mg L^{-1} exhibited 98 per cent mortality against third instar larvae of *An. culicifacies* up to sixth week. It expressed reduced ovipositional deterrent activity by 89 per cent.

2.4.1.2.5.1 Carrier materials for tablet preparation

Baghel *et al.* (2014) formulated charcoal, talc and chalk based tablets of *Trichoderma viride* Pers at 15% that exhibited 7.7 x 10^8, 5 x 10^7 and 0 cfu g^{-1}, at 260 days after storage, and selected charcoal as the superior carrier material.

Gola *et al.* (2019) formulated *B. bassiana* tablets in rice flour and reported 52.5 per cent multimetal removal ability even after 12 months of storage.

2.4.1.2.5.2 Binding agents used for tablet preparation

de Medeiros *et al.* (2005) formulated *L. sphaericus* tablets with 3 % polyvinyl pyrrolidone as binder which exhibited good binding properties. According to Sathyasree *et al.* (2008), *B. bassiana* tablets can be formulated in di calcium phosphate with the 10 % microcrystalline cellulose as an excellent binding agent.

Tekade *et al.* (2011) observed that use of more than 10 % concentration of acacia catechu gum as binder, affected the flow characteristics of the tablet with increased disintegration time. Raypuriya *et al.* (2019) examined the compatibility of talc with various adjuvants and found that carboxy methyl cellulose could effectively be used as a binder for tablets as it showed maximum radial growth (66.5 mm) with least growth inhibition (7.16 per cent) of *M. anisopliae*.

2.5 SHELF LIFE OF MICROBIAL FORMULATIONS

Shelf life of a mycopesticide at ambient temperature is an essential requirement for acceptance and commercialization of the formulations (Burges and Jones 1998)

B. bassiana conidia were formulated as dustable powder (DP), oil suspension (OS) and as rapeseed oil pellet. Pellet and DP recorded high conidial viability of 84.7 and 83.3 per cent where as in OS it was low (55.3per cent) after 45 days of storage (Hidalgo *et al.*, 1997).

Bti tablets kept under normal storage conditions remained viable for 24 months of storage and exhibited 97 per cent mortality against *Culex* larvae (Aroujo *et al.*, 2007). Conidial viability in the *B. bassiana* tablet formulation decreased over time, depending on storage temperature. Tablet formulation tested at 25° C contained 90 per cent viable conidia for 1 h, whereas conidia stored at lower temperatures remained viable over 18 months (Satyasree *et al.*, 2008). Gerding-Gonzalez *et al.* (2013) formulated sodium alginate pellets of *B. bassiana* with chitin at varying levels and suggested that while incorporating chitin 2%, the formulation exhibited longer shelf life and lesser contamination by saprophytic fungi.

Different volcanic materials were used by Victoria *et al.* (2016) as carrier for studying the effect of temperature and viability on *B. bassiana*. They selected Puyehue pumice and paloblanco as best carriers that maintained high viability of 70.8 ± 4.0 and 70.2 ± 2.5 per cent after 30 days of storage, whereas the viability was 65.9 ± 3.3 and 69.5 ± 1.7 per cent after exposure to 50^0C.

Increase in shelf life of *M. rileyi* WP formulation was observed when adjuvants were added along with kaolinite. Viability varied from 31.33 to 5.33 x 10^8 and 30.67 to 0.0 x 10^8 cfu mL^{-1} spore in formulations with and without adjuvants (Patil and Jadhav, 2019).

2.6 MICROBIAL FORMULATIONS FOR MOSQUITO CONTROL

2.6.1 EPB Formulations

Bti and *L. sphaericus* are the main entomopathogenic bacteria available as formulations for mosquito control in the market.

Bourgouin *et al.* (1984) formulated *L. sphaericus* RB 80, as powder using talc as carrier which was highly effective against *An. stephensi* and *Cx. pipiens* and the larvicidal potency remained high even after exposure to heat indicating an excellent stabilty.

Encapsulation of *L. sphaericus* 2362 with calcium alginate tested against *Culex* sp. larvae showed that encapsulation gave spore stability, more resistance to UV and increased persistence against high temperature (Elcin, 1995).

Application of *Bti* aqueous suspension Vectobac 12AS @ 1.21 L ha^{-1} when tested against *Cx. quinquefasciatus* exhibited more than 92.5per cent mortality, 48 h after spraying (Amalraj *et al.*, 1999).

Benjamin *et al.* (2005) formulated VectoBac DT, a tablet formulation of *Bti* and evaluated for the potential in controlling vectors of dengue fever and reported 90 per cent control of *Ae. aegypti*. Vectobac WDG formulation based on *Bti* strain AM65-52 was effective against temephos resistant *Ae. aegypti* for a period of three months with 48 per cent reduction in dengue fever (Setha *et al.*, 2016).

Fly ash based water dispersible powder formulation of *Bti* was found to effective against *Cx. quinquefasciatus, Ae. aegypti and An. stephensi* larvae with LC_{50} values 0.0417, 0.0462 and 0.1091 mg^{-1}, respectively (Tamilselvan *et al.*, 2017).

2.6.2 EPF Formulations

Traditional formulations of EPF for mosquito control include wettable powder and emulsifiable concentrate. Albernaz *et al.* (2009) developed sunflower oil-based formulations of *M. anisopliae* IP 46 that showed ovicidal action against *Ae. aegypti.* When treated with oil-in-water formulated propagules in 10 per cent oil, eclosion was 13.7 per cent, and when conidia were put in pure oil, eclosion was entirely blocked.

Bukhari *et al.* (2011) developed an oil formulation of *B. bassiana* with shellsol T as carrier and tested it against *Anopheles* larvae in the laboratory and in the field. The formulation when sprayed on small meshed cotton nets was found to be effective carrying 90 per cent mortality in *An. gambiae,* when exposed for 30 min (Farenhorst *et al.,* 2011).

Granular formulation of *M. anisopliae* JEF-003 showed 73 per cent mortality against *Ae. albopictus* larvae, 2 days post inoculation and 90 per cent mortality after 5 days post inoculation and suggested that it can be practically used in the mosquito management (Lee *et al.,* 2015).

Rodrigues *et al.* (2021) prepared microcrystalline cellulose and diatomaceous earth based *Metarhizium humberi* IP 46 MS granules and suggested that 93 to 96.5 per cent relative humidity was critical for the development of the fungus. These granules caused 90 per cent death of adult *Ae. aegypti* within six days of treatment and suggested its potential for mosquito control.

2.6.3 Other Microbial Formulations

Buchatsky *et al.* (1987) developed the first viral formulation Viroden for mosquito control which was based on *Aedes* densonucleosis virus. Laboratory studies demonstrated the effectiveness of the Viroden against the immature stages of *Ae. aegypti* causing 77 per cent reduction in population.

Encapsulated formulation of *S. carpocapse* DD-136 showed high mortality against *Ae. aegypti* larvae (Welch and Bronskill, 1962).

Sand formulation of the protozoa *Chilodonella uncinata* BP 610- 2016 @ 3.5 x 10^4 cells mL^{-1} in "infusion bag" methodology resulted in 100 per cent control of

Ae. aegypti larvae for 8 to 9.5 weeks in the breeding habitat (Das, 2019). The formulation was reported to have a shelf life of more than 18 months.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled “Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control” was carried out in the Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani during 2019 – 2021.

3.1 PATHOGENICITY STUDIES

Infectivity of entomopathogenic fungi (EPF) such as *Metarhizium anisopliae* (Metsch.) Sorokin, *Beauveria bassiana* (Bals.) Vuillemin, *Lecanicillium lecanii* (Zimmerman) Zare and Gams and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno (Plates 1a to1e) to the adults and larvae of mosquitoes was carried out under laboratory conditions.

Mosquito larvae were collected from three different locations of Thituvananthapuram district and identified up to the genus level using pictorial representations and morphological characters of larvae as described by Littig and Stojanovich (1990). They were identified as *Anopheles, Aedes* and *Culex* (Plates 2 to 4).

3.1.1 Maintenance of Fungal Cultures

The fungi used for the study were those maintained at Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani. *M. anisopliae* (Ma4), *B. bassiana* (Bb5) and *L. lecanii* (Vl 8) were originally sourced from National Bureau of Agricultural Insect Resources (NBAIR) and *B. bassiana* ITCC 6063 and *L. saksenae* ITCC 7714, were the isolates from the Department of Agricultural Entomology, College of Agriculture, Vellayani. Cultures used for the study were revived periodically by passing through susceptible hosts insects to maintain their virulence. Pure and sub cultures of these fungi were maintained in Potato Dextrose Agar (PDA) slants.

3.1.2 Mass Multiplication of Fungi

The fungi were mass produced in Sabouraud Dextrose Broth (SDB) by static liquid fermentation method. Medium for maintaining fungal cultures was prepared by dispensing 40 g dextrose and 10 g peptone in 1 L of water. A quantity of 100 mL the medium was poured to 250 mL conical flasks. The flasks were then plugged with cotton and sterilized at 121^0C and 1.1 kg cm^{-2} for 20 min in a horizontal autoclave. The medium was cooled to room temperature and inoculated aseptically with spore suspensions of pure cultures mentioned in para 3.1.1.

3.1.3 Maintenance of Mosquito Culture

Mosquito larvae were reared as per the guidelines given by FAO (2017). Larvae of all the three species were maintained separately in tap water (1.5 L) taken in plastic trays of dimension 40 × 30 × 8 cm (Plate 5) and kept undisturbed for 24 h, to dissipate chlorine. Collected larvae were then transferred to the trays using a fish net with handle. The trays were then covered with a transparent polythene sheet to avoid evaporative cooling of larval water, to maintain a constant temperature and prevention of oviposition by other mosquitoes or insects. A pinch of aquarium fish food (tetramin bits) was provided as feed. A little quantity of water was removed daily which was replenished with the equal quantity of tap water dissipated of chlorine, to prevent scum formation in the larval water. Pupae were separated from larvae by removing them with a plastic pipette and transferred into new containers. The culture was maintained in such a way that there is a continuous supply of test insects for the laboratory experiments.

3.1.4 Pathogenicity Tests

Pathogenicity of the test fungi was carried out in the adult as well as larvae of *Anopheles, Aedes* and *Culex* in the laboratory, under ambient conditions. Spore suspensions were prepared by blending 14 day old cultures of *M. anisopliae* and *B. bassiana*, and 21 day old cultures of *L. lecani* and *L. saksenae* in a blender and filtered through a double layer muslin cloth. Spore count was determined as 10^8 spores mL^{-1} for *M. anisopliae* and *B. bassiana* and 10^7 spores mL^{-1} for *L. lecaniii* and *L. saksenae* using a Naubauer haemocytometer.

1a. *Metarhizium anisopliae* Ma4

1b. *Beauveria bassiana* Bb5

1c. *Beauveria bassiana* Bb 6063

1d. *Lecanicillium lecani* Vl 8

1e. *Lecanicillium saksenae* LsVs 7714

Plate 1. Cultures of entomopathogenic fungi

2a. Larva

2b. Adult

Plate 2. *Anopheles* sp.

3a. Larva

3b. Adult

Plate 3. *Aedes* sp.

4a. Larva

4b. Adult

Plate 4. *Culex* sp.

5a. Plastic tray containing larvae

5b. Larval feed

5c. Fish net with handle

Plate 5. Rearing facilties

3.1.4.1 Larvae

Pathogenicity was tested by introducing 10 larvae of fourth instar (seven day old and uniform sized) into 100 mL tap water devoid of chlorine taken in plastic cups of size 8 x 4 cm (Plate 6). Larvae were fed daily once with tetramin bits. The experiment was carried out in CRD with seven treatments and three replications. Spore suspension (2 mL) of each of the fungus was inoculated to these. The treatments were as follows

T1 - *M. anisopliae* – NBAIR isolate Ma4 (10^8 spores mL^{-1})

T2 - *B. bassiana* – NBAIR isolate Bb5 (10^8 spores mL^{-1})

T3 - *B. bassiana* – KAU isolate ITCC 6063 (10^8 spores mL^{-1})

T4 - *L. lecanii* – NBAIR isolate Vl 8 (10^7 spores mL^{-1})

T5 - *L. saksenae* – KAU isolate ITCC - 7714 (10^7 spores mL^{-1})

T6 - *Bacillus thuringiensis* var. *israelensis* formulation 5% - biocontrol check

T7 - Malathion 50 EC 0.1% - chemical check

T8 – Untreated control

The treated larvae were observed for symptoms of mycosis and mortality at 24 h interval

3.1.4.2 Adults

From the laboratory culture, pupae were manually separated @ 10 per container by picking them one by one with a plastic pipette and transferred to plastic containers of size 15x 6 cm having caps with little quantity of water (Plate 7). A circular opening on the lid of the container served as the food inlet for the emerging adults. Upon adult emergence (two to three days) the adults were fed with 10% sugar solution by dropping a cotton ball soaked in the sugar solution. The adults were treated with five mL of the spore suspensions of each of the fungus at their respective doses, by spraying onto the inner walls of the container, using an atomizer. The treated insects were observed for symptoms of mycosis and mortality at 24 h interval.

3.1.4.3 Determination of effective dose

Effective dose of the fungus was estimated for each of the mosquito species by studying dose mortality response of larvae treated with varying concentrations of spore suspension of the fungus ranging from 10^6 to 10^{10} spores mL^{-1}. Spore suspensions were prepared as described in 3.1.4. and treated as per the procedure described in 3.1.4.1. Each treatment was replicated four times with 10 insects per treatment. Observations on mortality were recorded at 12 h interval for a period of four days.

3.1.4.4 Determination of lethal concentrations and lethal time

Mortality data recorded as in para 3.1.4.3 was made use of for calculating the Lethal Concentrations (LC_{50} and LC_{90}) and Lethal Time (LT_{50} and LT_{90}).

3.1.4.5 Susceptibility of different stages

Fourth instar larvae, pupae and adults were taken in different containers and treated with the most effective fungus @ 10^8 spores mL^{-1}. Larvae were fed with tetramin bits whereas adults were fed with 10% sugar solution. Observations on mortality were recorded at 24 h interval for a period of four days.

3.2 DEVELOPMENT OF WATER DISPERSIBLE TABLETS FOR THE MANAGEMENT OF MOSQUITO LARVAE

The most effective entomopathogenic fungus selected from the above experiment was selected for formulation studies.

3.2.1 Standardization of Carrier Material

Carrier materials tested for formulating the tablets were T1 - talc, T2- bran, T3- talc + chitosan, T4- bran + chitosan. Carrier : chitosan ratio in T3 and T4 was fixed by the trial and error method. Conidial germination was observed at weekly intervals for a period of five weeks and mortality caused in larvae was noted.

The experiment was carried out in CRD with four treatments and four replications.

6 a. 100 ml tap water

6 b. Plastic container with larvae

6c. Inoculation

Plate 6. Treatment method - Larvae

7a. Container with pupae

7b. Emerging adults feeding with10% sucrose

7c. Inner surface of container

7d. Adult on the wall of container

Plate 7. Treatment method - Adults

3.2.1.1 Conidial germination

Conidial germination assay was carried out by ESLAQ method (Oliveria *et al.*, 2015). From each treatment 0.1 g was added to sterile test tubes containing 5 mL of 0.05% tween 80 and vortexed for three min. The concentration was adjusted to 1.0^{-5} spores mL^{-1} by serial dilution technique and 0.3 mL of this suspension was dropped onto a sterile glass slide provided with a thin layer of PDA. Slides were kept in a moist chamber prepared using a sterile petriplate of 9 cm lined with moistened filter paper. Slides were places over two sterile tooth picks placed over the moistened filter paper. The entire unit was sealed with cling film and incubated at 27 °C in darkness for 24 h. The slides were examined for conidial germination under 40 X in a compound microscope. Conidia were counted as germinated if the length of the germ tube had at least twice the diameter of the spore. Viability was calculated using the formula

$$\text{Germination percentage} = \frac{\text{No. of germinated conidia}}{\text{Total No. of conidia counted}} \times 100$$

3.2.1.2 Mortality

Fourth instar larvae (10 no.) were taken in plastic containers of size 8 x 4 cm with 100 mL of water and were fed with tetramin bits. After 24 h, the larvae were treated as described in the experiment 3.2.1. Mortality was observed at 24 h interval for a period of five days.

3.2.2 Standardization of Binding Agent

Superior carrier material selected from experiment 3.2.1 was used for standardising suitable binding agents. The binding agents tested were Carboxy Methyl Cellulose (CMC), Polyvinyl Pyrrolidone (PVP), Acacia Gum Arabic (AG) and Microcrystelline Cellulose (MCC) and the ratio of carrier material : binding agent was determined by trial and error method. Experiment was carried out with five treatments and four replications in CRD. Observations were recorded on conidial germination and mortality as mentioned in para 3.2.1

3.3 SHELF LIFE OF THE TABLETS

To study the shelf life, tablets were prepared using the standardized carrier and binding agents from the above experiments. Tablets used for the shelf life studies were prepared using a tablet press.

3.3.1 Fabrication of Tablet Press

A tablet press is a mechanical device that compresses powder into tablets of uniform size and weight. The essential parts of a tablet press are die plate, cavity plate, pegs, guiding pins and holes. It was fabricated in stainless steel which consisted of two plates, the lower die plate fixed with stainless steel pegs of uniform diameter and an upper cavity plate having holes of uniform diameter (Plate 8).

The die plate was fabricated in stainless steel of 5 mm thickness and a dimension of 15 x 15 cm. Pegs of 1.5 cm (20 no.) were fixed with the help of a computer numerical control machine and welding machine, which served as the die rollers. Two additional pegs of diameter 1.5 cm and height 2.5 cm were fixed on either side of the plate to serve as the guiding pins. The lower cavity plate was also fabricated in stainless steel of thickness 10 mm and dimension of 15 x 15 cm. Holes of 1.7 cm diameter (20 no.) were drilled on this plate coinciding with the pegs on the die plate, so that on pressing the upper cavity plate pasted with the tablet material over the lower die plate, uniform sized tablets were cut off from it. Two holes were additionally drilled on this plate on either side corresponding to the position of guiding pins fixed on the lower plate to hold the guiding pins. The apparatus was polished for a fine finishing.

3.3.2 Formulation of Tablets

3.3.2.1 Preparation of spore pellets

Spore suspension of the fungus was prepared as mentioned in para 3.1.4. The resultant spore suspension was centrifuged in a Rotek centrifuge at 4000 rpm for 20 min. The supernatant was then decanted to get spore pellets. The spore count was enumerated as 10^{10} spores mL^{-1} using a Neubaur haemocytometer.

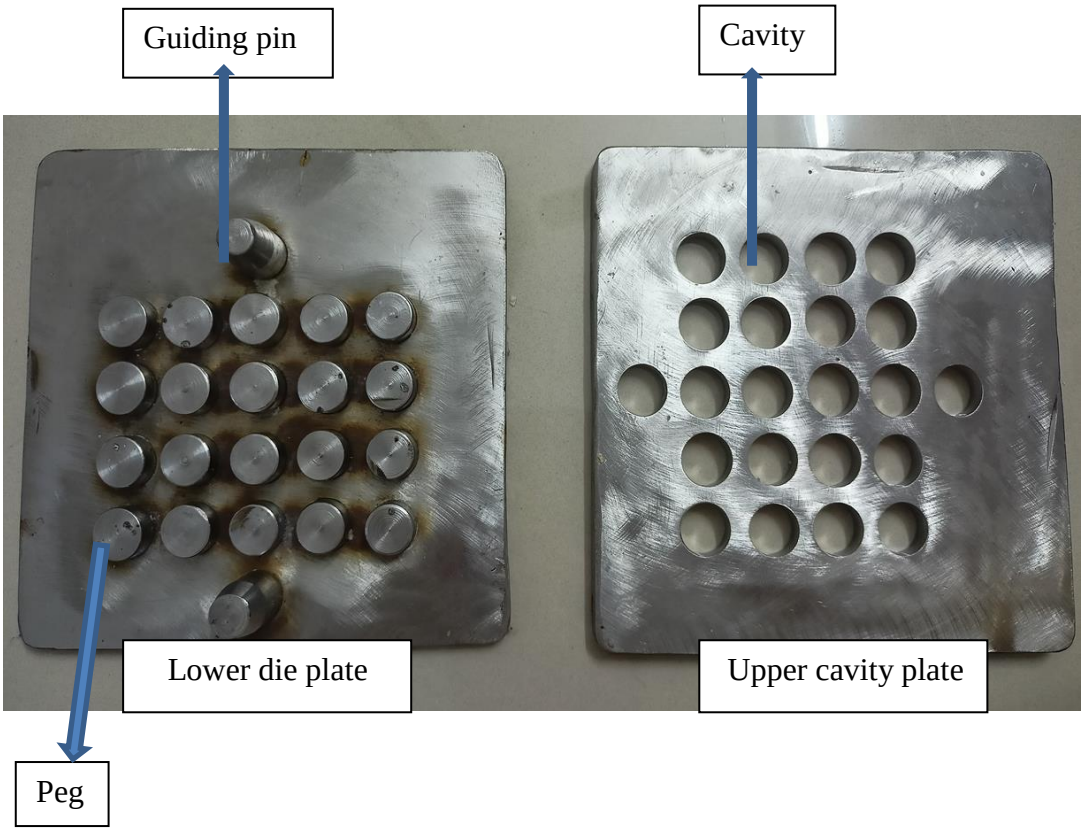


Plate 8. Tablet press

3.3.2.2 Preparation of water dispersible tablets

Fixed quantity of the superior carrier material (3.2.1) was mixed with the most suitable binding agent (3.2.2). To this the fungal conidia @ 10^{10} spores mL^{-1} was added in the ratio 75: 25. It was made into a thick paste (Plate 9) by using sterile water as diluent. This paste was then loaded in the tablet press (Plate 10) and compressed to form tablets (Plate 11) of uniform weight (one g).

3.3.3 Shelf Life of Tablets at Varying Moisture Levels

Observations were recorded on conidial germination to assess viability at fortnightly intervals for a period of three months and mortality at monthly interval to assess virulence as mentioned in para 3.2.1. Tablets were simultaneously tested to determine the ideal moisture content. The moisture content tested was eight, 10 and 15 per cent, by adding the desired levels of water while preparing the tablet paste. Five tablets from each lot were powdered and the moisture content was determined using a Moisture analyser AXIS model ATS 60. Experiment was carried out with three treatments and five replications, in CRD design.

3.3.4. Determination of the extent of contamination

Contamination by other fungi was assessed by estimating the colony forming units (cfu). Cfu was estimated by dilution plate method. One gram of tablet was dissolved in nine mL of sterile water and was serially diluted to 10^{-5} concentration. This was poured onto a sterile petriplate. To this 15 mL molten PDA was poured and gently rotated for uniformly spreading the spore suspension. The plates were then incubated at room temperature. Number of cfu was estimated after two weeks of storage.

$$\text{No. of cfu} = \frac{\text{No. of colonies x dilution factor}}{\text{Weight of sample}}$$

3.3.5 Physical characterization of tablets

3.3.5.1 Thickness

Thickness of the tablets was measured using digital vernier caliper.

3.3.5.2 Disintegration time

Disintegration time was estimated using a disintegration test apparatus (Plate 12a). The apparatus consisted of six uniform tubes which has an up and down motion in the disintegration medium maintained at 30^0C. One tablet each was kept in the all the six tubes. Time taken for all the six tablets to break down and pass through the mesh at the bottom of the tube was noted.

3.3.5.3 Friability

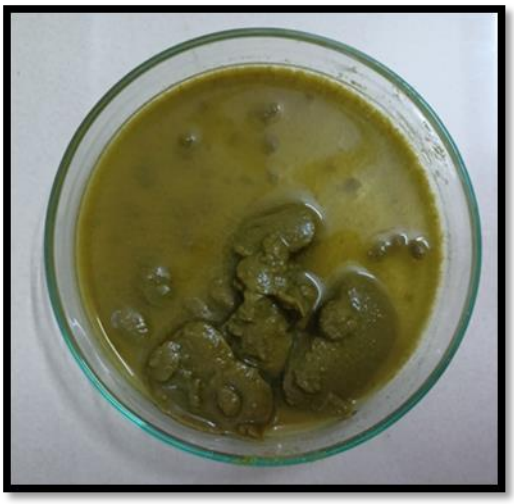
Tablets tested for friability using a friability test apparatus (Plate 12b). Initial weight of tablets (10 No.) was recorded collectively, before placing them in the unit, which was then operated for 10 min at 30 rev min^{-1}. The tablets were then reweighed and the loss in weight was calculated.

3.4 EFFICACY OF TABLETS IN MANAGING MOSQUITO LARVAE

Tablets prepared as per the above standards were tested for their efficacy under laboratory conditions and field conditions against mosquito larvae and the dosage was standardized.

3.4.1 Effect on Mortality and Emergence of larvae

A preliminary experiment was carried out under laboratory conditions. Batches of 100 fourth instar healthy larvae were transferred by means of fish nets to disposable containers each containing 1 L of tap water (Plate 13a). Four replications were set up for each treatment and equal number of larvae maintained in untreated water served as control. They were fed daily with tetramin bits, till the end of the experiment. After 24 h, tablets were added to each container @ one, two, three and four L^{-1}. Mortality was recorded at 24 h interval until all of them have pupated. Larvae that pupated during the experiment (Plate 13b) were removed from the



9a. Spore pellet

9b. Mixing of carrier and binding agent with spore pellet

9c. Tablet paste

Plate 9. Tablet paste preparation

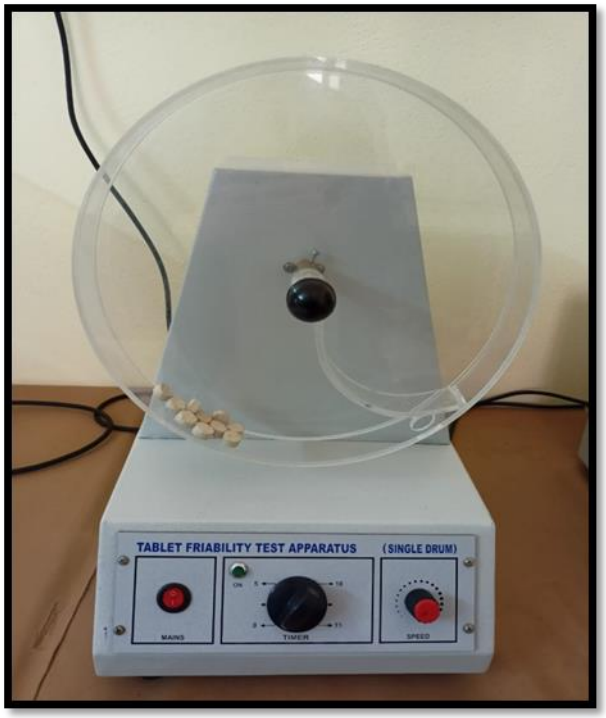
10a. Loading of tablet paste in cavity plate

10b. Tablet paste compressed to tablets

Plate 10. Tablet making

Plate 11. *Metarhizium anisopliae* tablets

12a. Tablet disintegration test apparatus



12b. Tablet friability test apparatus

Plate 12. Tablet physical properties testing apparatus

container and transferred to another container for evaluating adult emergence (Plate 13c). Percentage adult emergence was calculated using the formula

$$\text{Percentage adult emergence} = [(T/C) \times 100]$$

T – Percentage survival or emergence in treated batches

C – Percentage survival or emergence in control

3.4.2 Field Level Treatment

Four containers carrying 10 L of tap water was taken and kept overnight for dissipating chlorine. Top of the containers were closed using mosquito net (Plate 14a) to avoid egg laying before dissipation of chlorine. After 24 h, nets were removed allowing the mosquitoes to lay eggs. After three days *Culex* egg rafts were observed in the containers (Plate 15). Hatching was noted within two to three days. Upon hatching larvae were fed daily with tetramin bits. Containers were then covered with mosquito nets to avoid further egg laying. Within seven days the larvae reached third or fourth instar, upon which the pre-treatment count was taken.

3.4.2.1 Assessment of Pre-treatment density

Larval and pupal abundance in the treatment and control containers were determined by "Flow in" method suggested by European Centre for Disease Prevention and Control (ECDPC, 2018), which is the technique recommended for shallow water (depth < height of the handle of the dipper). A dipper with 1 L capacity was used for the sampling (Plate 14b). The bottom of the dipper was pushed into the water which enables larvae to flow into the dipper.

$$\text{No. of larvae} = \frac{\text{Total No. of larvae in n dips}}{n} \times 10$$

Where n is No. of dips taken from the sample.

3.4.2.2 Treatment

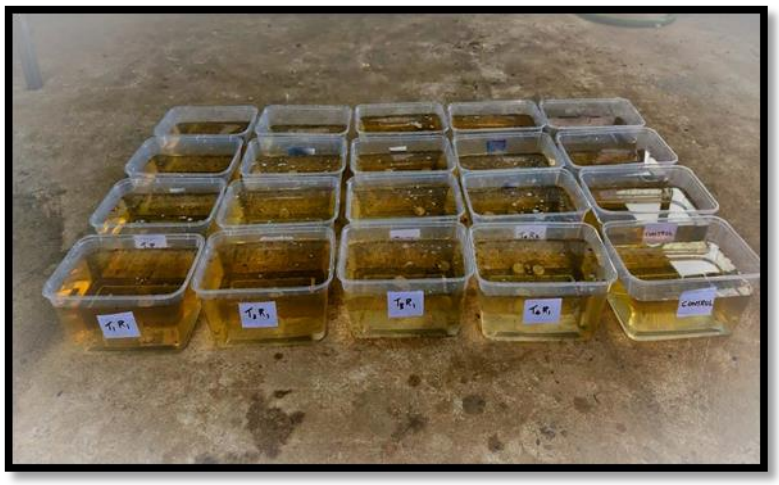
Each container was treated with tablets of varying doses (seven, eight, nine and 10 tablets). Experiment was carried out in CRD with four treatments and four replications.

3.4.2.3 Assessment of post treatment density

Post treatment density was also assessed by the same procedure in 3.4.2.1 by counting dead larvae collected in the dip. The impact of treatments on the larvae was evaluated by counting the dead larvae in the samples collected on the 4^{th}, 7^{th} and 10^{th} day after treatment.

3.5 STATISTICAL ANALYSIS

The data were subjected to analysis of variance (ANOVA) and the treatment differences were compared. Probit analysis was carried out to work out LC_{50}, LC_{90}, LT_{50} and LT_{90} with confidential limits fixed at 95 per cent. The software used was General R – shiny based Analysis Platform Empowered by Statistics (GRAPES), developed by Kerala Agricultural University.



13a. Larvae treated with tablets

13b. Adult emergence from seperated pupae

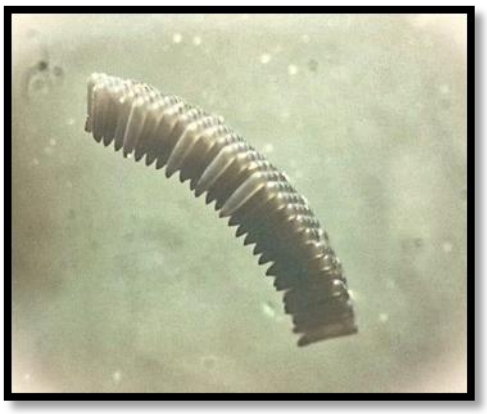
13c. Mosquito cage for adult Emergence

Plate 13. Laboratory level treatment

14a. Containers closed with mosquito nets

14b. Dipper with 1 L capacity

Plate 14. Field level treatment



15a. Unhatched egg raft

15b. Hatched egg raft

15c. Larva

15d. Pupa

15e. Adult

Plate 15. Different stages of *Culex* mosquito

Results

4. RESULTS

Results of the thesis entitled “Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control” carried out during 2019-21 in the Department of Agricultural Entomology, College of Agriculture, Vellayani is depicted below. The work was conducted under four main modules *viz.*, pathogenicity of entomopathogenic fungi to mosquitoes, development of water dispersible tablets for the management of mosquito larvae, shelf life studies and assessment of efficacy of the tablets in managing larvae.

4.1 PATHOGENICITY OF ENTOMOPATHOGENIC FUNGI TO MOSQUITOES

Results of pathogenicity test conducted using the entomopathogenic fungi (EPF) *Metarhizium anisopliae* (Metsch.) Sorokin NBAIR isolate Ma4, *Beauveria bassiana* (Bals.) Vuillemin NBAIR isolate Bb5, *B. bassiana* KAU isolate ITCC 6063, *Lecanicillium lecanii* (Zimmerman) Zare and Gams NBAIR isolate Vl 8 and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno KAU isolate ITCC - 7714 to the common mosquito genera *Anopheles*, *Aedes* and *Culex*, is presented below.

4.1.1 Symptoms of Mycosis

Disease symptoms observed in larvae and adults of different mosquito species to EPF infection is given below.

4.1.1.1 M. anisopliae

4.1.1.1.1 Larvae

Larvae treated with spore suspension of Ma 4 @10^8 spores mL^{-1} exhibited similar symptoms in all the species. After 12 h of treatment, infected larvae became less active and exhibited sinking movement, whereas uninfected ones always stayed on water surface. *Aedes* larvae normally exhibit wriggling motion, but once infected with Ma4, the wriggling motion was arrested and turned morbid. Mortality was observed after 24 h of treatment in all the species at varying degrees. Dead larvae after three days showed copious amount of mucus around the body and larval colour

changed from dark grey to white. Degeneration of gut was observed in the dead larvae when viewed under a binocular stereo microscope at 10 X magnification (Plate16).

4.1.1.1.2 Adults

Adults of all the three species were susceptible to Ma4 and exhibited similar symptoms of infection. They became inactive after 24 h of treatment and were found idle on the walls of the container, of which few were dead, mummified and attached to the walls of the container. After 24 h of mortality, white mycelial growth of the fungus was observed. After 72 h of death, fungi started sporulating and green spores were observed all over the cadaver (Plate 17).

4.1.1.2 B. bassiana isolates

4.1.1.2.1 Larvae

Larvae treated with spore suspensions of Bb5 and Bb 6063 @10^8 spores mL^{-1} exhibited similar symptoms as that of Ma4. But time taken for mortality varied.

4.1.1.2.2 Adult

Spores of Bb5 and Bb 6063 were infective to adults of all species of mosquito treated. Symptoms of infection resembled that of Ma4, but, when it was placed in moisture chamber, there was no mycelial growth on the surface of cadaver as in the case of Ma4 infected adults.

4.1.1.3 L. lecanii

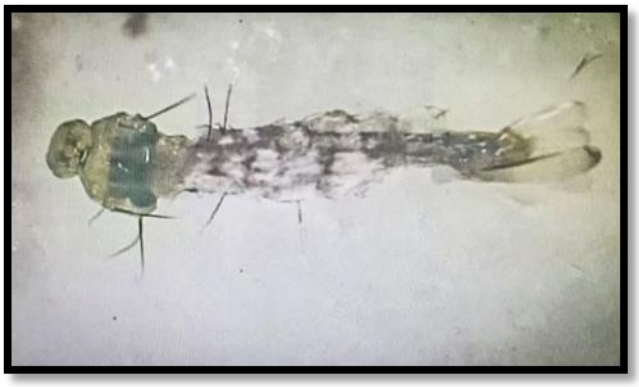
4.1.1.3.1 Larvae

Spores of Vl 8 were infective to all species of larvae at varying levels. *Culex* exhibited highest mortality. Symptoms observed were similar to that of Ma4.

4.1.1.3.2 Adults

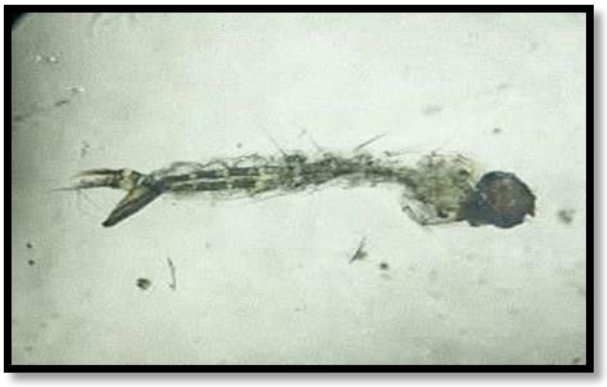
Mortality caused by Vl 8 to the adults was very less when compared that of larvae. Even though the fungus was infective, no mycelial growth was observed in cadavers.

4.1.1.4 L. saksenae 7714



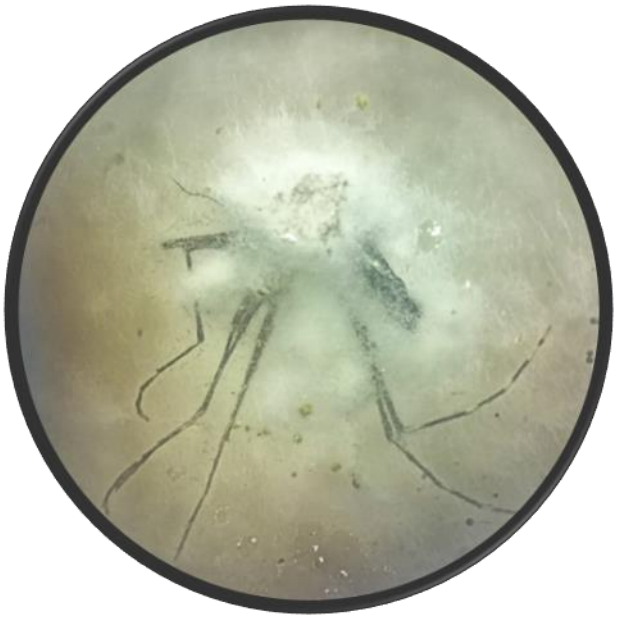
16a. *Anopheles*

16b. *Aedes*



16c. *Culex*

Plate 16. Degeneration of gut in the infected larvae after *Metarhizium anisopliae* treatment (10 X)



17a. White mycelial growth

17b. Sporulation on *Aedes* sp.

Plate 17. Symptoms of mycosis caused *by Metarhizium anisopliae* in *Aedes* sp.

4.1.1.4.1 Larvae

The spore suspension of the test fungus was infective to larvae only to a lesser extent. More than 80 per cent of treated larvae pupated after five days of treatment (DAT). No mucilage was observed on the surface of the dead larvae as in the case of other fungi.

4.1.1.4.2 Adults

Adults exhibited very less mortality and no mycelial growth was observed in the infected cadavers.

4.1.2 Mortality of Test Insects

Both the adults and larvae of *Anopheles, Aedes* and *Culex* were found to be infected by all the five fungi tested.

***4.1.2.1 Anopheles* sp.**

4.1.2.1.1 Larvae

Data on mortality of larvae taken at 24 h interval is presented in Table 1. One day after treatment, highest mortality was observed with *M. anisopliae*, causing 23.33 per cent mortality. The effect of both the isolates of *B. bassiana* (Bb5 and Bb 6063) was similar, causing 13.33 per cent mortality. Least mortality was observed in *L. lecanii* (3.33 per cent), while in the biocontrol check *Bacillus thurunguensis israelensis, Bti* (green milestone) 5% formulation mortality was high (33.33 per cent). Corresponding mortality noted in chemical check (malathion 50 EC), was 100 per cent.

After two days of treatment, the trend observed was exactly the same. Among the test fungi, highest mortality was noted with Ma4, which was on par with Bb5 (43.33 per cent each). The mortality caused by Bb5 was similar to that observed with Bb 6063 (33.33 per cent and 20 per cent respectively). Least mortality was recorded with Vl 8 and LsVs 7714 causing 6.66 per cent in each.

Table 1. Efficacy of entomopathogenic fungi to *Anopheles* sp. larvae

Treatments	Cumulative mortality of 4th instar larvae (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	23.33^{bc} (28.78)	43.33^{b} (41.15)	70.00^{b} (56.99)	86.66^{b} (68.85)	96.66^{a} (83.66)
Bb 5 @ 10^8 spores mL^{-1}	13.33^{c} (21.14)	33.33^{bc} (35.21)	43.33^{c} (41.15)	76.66^{b} (61.22)	83.33^{b} (66.14)
Bb 6063 @ 10^8 spores mL^{-1}	13.33^{cd} (17.80)	20.00^{c} (26.07)	43.33^{c} (40.77)	56.66^{c} (48.93)	60.00^{c} (51.14)
Vl 8 @ 10^8 spores mL^{-1}	3.33^{de} (6.33)	6.66^{d} (12.38)	13.33^{d} (17.31)	23.33^{d} (21.14)	33.33^{d} (35.21)
LsVs 7714 @ 10^8 spores mL^{-1}	0^{e} (0.28)	6.66^{d} (12.38)	13.33^{d} (21.14)	13.33^{d} (21.14)	16.66^{d} (23.36)
Bti 5% – biocontrol check	33.33^{b} (35.21)	43.33^{b} (41.15)	53.33^{bc} (46.92)	63.33^{c} (52.77)	73.33^{bc} (59.00)
Malathion 50 EC 0.1% – chemical check	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)
Untreated control	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)
CD (0.05)	(12.067)	(10.751)	(13.904)	(7.866)	(11.898)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

On the third day, 70 per cent mortality was observed with Ma4 while it was 43.33 per cent in Bb5 and Bb 6063. Mortality was very less (13.33 per cent) in Vl 8 and LsVs 7714. The corresponding mortality observed with *Bti* was 53.33 per cent which was superior to Vl 8 and LsVs 7714.

At fourth day after treatment (DAT), effect of *M. anisopliae* was on par with that of Bb5 with a mortality of 86.66 per cent and 76.66 per cent respectively. This was followed by Bb 6063 with 56.66 per cent mortality which was on par with *Bti* (63.33). Vl 8 and LsVs 7714 exhibited lowest mortality of 23.33 and 13.33 per cent, respectively and was found to be on par with each other.

At the end of the experiment also *M. anisopliae* was found to be superior to all other treatments causing 93.33 per cent mortality, followed by Bb 5 (83.33 per cent). Bb 6063 caused 60 per cent mortality which was statistically lower than the former treatments but higher than those recorded with Vl 8 and LsVs 7714 (33.33 per cent and 16.66 per cent respectively). *Bti* caused significantly high mortality of 73.33 per cent which was similar to the treatments of Bb5 and Bb 6063.

4.1.2.1.2 Adults

Pathogenicity of different EPF to adults of *Anopheles* is furnished in Table 2.

Twenty four Hours After Treatment (HAT), mortality observed was negligible (0-3.33 per cent) with all the test fungi. Ma4 caused 3.33 per cent mortality which was on par with that of biocontrol check *Bti.* There was 100 per cent death in malathion 50 EC treatment.

At 48 HAT, highest mortality of 23.33 per cent observed with Ma4, which was on par with the mortality observed with Bb5 (16.66 per cent) and *Bti* (13.33 per cent). Both Bb 6063 and Vl 8 were on par, recording 3.33 per cent each. There was no mortality of adults when treated with LsVs 7714.

Table 2. Efficacy of entomopathogenic fungi to adult *Anopheles* sp.

Treatments	Cumulative mortality (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	3.33^{b} (6.80)	23.33^{b} (28.78)	46.66^{b} (43.08)	73.33^{b} (59.00)	83.33^{b} (66.15)
Bb 5 @ 10^8 spores mL^{-1}	0^{b} (0.91)	16.66^{b} (23.86)	26.66^{c} (30.79)	43.33^{c} (41.07)	56.66^{c} (48.93)
Bb 6063 @ 10^8 spores mL^{-1}	0^{b} (0.91)	3.33^{c} (6.80)	6.66^{d} (12.59)	16.66^{de} (23.36)	23.33^{d} (28.29)
Vl 8 @ 10^8 spores mL^{-1}	0^{b} (0.91)	3.33^{c} (6.80)	13.33^{d} (21.15)	16.66^{de} (23.86)	20.00^{de} (26.07)
LsVs 7714 @ 10^8 spores mL^{-1}	0^{b} (0.91)	0^{c} (0.91)	0^{e} (0.906)	6.66^{e} (12.59)	10.00^{e} (15.30)
Bti 5% – biocontrol check	3.33^{b} (6.749)	13.33^{b} (21.15)	33.33^{bc} (35.01)	56.66^{bc} (48.93)	66.66^{bc} (54.78)
Malathion 50 EC 0.1% – chemical check	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)
Untreated control	0^{b} (0.91)	0^{b} (0.91)	0^{e} (0.91)	0^{f} (0.91)	0^{f} (0.91)
CD (0.05)	(8.759)	(9.937)	(9.532)	(11.249)	(12.441)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

At 72 HAT, Ma4 recorded highest mortality of 46.66 per cent, which was similar to *Bti* (33.33 per cent). This was followed by the next superior treatment of Bb5 recorded 26.66 per cent mortality. Both Bb 6063 and Vl 8 was found to be on par with each other causing mortality of 6.66 and 13.33 per cent. No mortality was observed in LsVs 7714 treated insects.

On the fourth day, 73.33 per cent death rate was observed with Ma4 which was superior to all other EPF treatments. Bb5 recorded 43.33 per cent mortality which was similar to *Bti* (56.66 per cent) and significantly higher than those observed with Bb 6063 and Vl 8 (16.66 per cent each). A negligible death rate of 6.66 was noted with LsVs 7714 treatment.

At the end of the experimental period (120 HAT), Ma4 resulted in 83.33 per cent death followed by Bb5 (56.66 per cent) and was found to be similar to *Bti* (66.66 per cent). Mortality of 23.33 per cent and 20 per cent was observed in treatments with Bb 6063 and Vl 8 which were on par. Least mortality of 10 per cent was recorded by LsVs 7714.

4.1.2.2 Aedes sp.

4.1.2.2.1 Larvae

All the fungi tested exhibited pathogenicity to *Aedes* larvae after 24 h of treatment (Table 3). Bb 6063 recorded highest mortality of 43.33 per cent, which was on par to that of Bb5 and the biocontrol check, *Bti* (26.66 per cent each). Mortality observed with Ma4 was 23.33 per cent which was superior to Vl 8 (10 per cent). Mortality noted in LsVs 7714 treated mosquitoes was 3.33 per cent. All the larvae treated with malathion 50 EC died at this point of time. There was no death in untreated control.

On the second day also, highest mortality of 53.33 per cent was observed in Bb 6063, which was found to be similar to those of Bb5, Ma4 and *Bti*, mortality being 43.33, 46.66 and 43.33 per cent, respectively. Mortality recorded in Vl 8 was 30 per cent which was significantly higher to that of LsVs 7714 (10 per cent).

Table 3. Efficacy of entomopathogenic fungi to *Aedes* sp. larvae

Treatments	Cumulative mortality of 4th instar larvae (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	23.33^{c} (28.78)	46.66^{bc} (43.07)	76.66^{b} (61.22)	98.66^{a} (83.66)	98.66^{a} (83.66)
Bb 5 @ 10^8 spores mL^{-1}	26.66^{bc} (30.99)	43.33^{bc} (41.15)	56.66^{c} (48.84)	66.66^{bc} (54.99)	70.00^{bc} (56.99)
Bb 6063 @ 10^8 spores mL^{-1}	43.33^{b} (41.15)	53.33^{b} (47.00)	56.66^{c} (48.93)	73.33^{b} (59.70)	76.66^{b} (61.71)
Vl 8 @ 10^8 spores mL^{-1}	10.00^{d} (15.09)	30.00^{c} (32.21)	46.66^{c} (43.07)	53.33^{c} (47.00)	60.00^{c} (50.85)
LsVs 7714 @ 10^8 spores mL^{-1}	3.33^{de} (6.33)	10.00^{d} (15.09)	13.33^{d} (21.14)	13.33^{d} (21.14)	13.33^{d} (21.14)
Bti 5% – biocontrol check	26.66^{bc} (30.99)	43.33^{bc} (41.07)	46.66^{c} (42.99)	60.00^{bc} (50.85)	66.66^{bc} (54.78)
Malathion 50 EC 0.1% – chemical check	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)
Untreated control	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)	0^{e} (0.28)
CD (0.05)	(11.385)	(14.222)	(11.022)	(12.462)	(10.450)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

On the third day, Ma4 was the superior treatment causing 76.66 per cent mortality. Mortality caused by both isolates of *B. bassiana* was 56.66 per cent each, while Vl 8 and *Bti* resulted in 46.66 per cent mortality which was on par. LsVs 7714 exhibited least mortality to *Aedes* larvae (13.33 per cent).

On the fourth DAT, mortality observed in Ma4 was on par with the chemical check malathion 50 EC (96.66 and 100, respectively). This was followed by Bb 6063 and Bb5, causing 73.33 and 66.66 per cent mortality, which was similar to the mortality exhibited by *Bti* (60 per cent). Vl 8 and LsVs 7714 recorded 53.33 and 13.33 per cent mortality, respectively.

The trend was similar on fifth DAT. No further mortality was observed in Ma4 treated insects, which was the superior treatment, followed by Bb 6063 (76.66 per cent). Bb5 caused 70 per cent mortality and was found to be on par with *Bti* (66.66 per cent). This was followed by Vl 8 which caused 60 per cent mortality. Least death rate was observed in larvae treated with LsVs 7714 (13.33 per cent).

4.1.2.2.2 Adult

Pathogenicity of different EPF to the adults of *Aedes* is depicted in Table 4. At 24 HAT, there was no mortality except in Ma4 (3.33 per cent), whereas malathion 50 EC exhibited 98.66 per cent mortality. The death rate observed with *Bti* was neglibible (6.66 per cent).

At 48 HAT, there was 26.66 per cent mortality in Ma4 which was on par with *Bti* (16.66 per cent) and Bb5 (13.33 per cent). Vl 8 and Bb 6063 were less pathogenic to test insects causing only 6.66 and 3.33 per cent mortality, which were statistically similar. No mortality was recorded with LsVs 7714, while malathion 50 EC resulted in complete mortality.

Mortality recorded at 72 HAT was 53.33 per cent when treated with Ma4 which was significantly similar to Bb5 that killed 36.66 per cent of test insects. Vl 8 recorded only 10 per cent mortality, which was lower than Bb5. A negligible death rate of 3.33 and 6.66 per cent was recorded in treatments with Bb 6063 and LsVs

Table 4. Efficacy of entomopathogenic fungi to adult *Aedes* sp.

Treatments	Cumulative mortality (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	3.33^{bc} (6.75)	26.66^{b} (30.79)	53.33^{b} (47.01)	83.33^{b} (66.64)	86.66^{b} (68.86)
Bb 5 @ 10^8 spores mL^{-1}	0^{c} (0.91)	13.33^{bc} (21.15)	36.66^{bc} (37.23)	53.33^{c} (47.01)	70.00^{c} (57.00)
Bb 6063 @ 10^8 spores mL^{-1}	0^{c} (0.91)	3.33^{de} (6.78)	6.66^{de} (12.59)	13.33^{de} (21.15)	23.33^{d} (28.78)
Vl 8 @ 10^8 spores mL^{-1}	0^{c} (0.91)	6.66^{cd} (12.59)	10.00^{d} (15.30)	16.66^{d} (23.86)	20.00^{d} (26.07)
LsVs 7714 @ 10^8 spores mL^{-1}	0^{c} (0.91)	0^{e} (0.91)	3.33^{de} (6.78)	6.66^{e} (12.59)	6.66^{e} (12.59)
Bti 5% – biocontrol check	6.66^{ab} (12.59)	16.66^{b} (23.85)	30.00^{c} (33.00)	50.00^{c} (45.00)	76.66^{bc} (61.22)
Malathion 50 EC 0.1% – chemical check	96.66^{a} (83.25)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)
Untreated control	0^{c} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{f} (0.91)	0^{f} (0.91)
CD (0.05)	(8.766)	(10.642)	(13.809)	(11.162)	(9.653)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

7714, which was similar. The corresponding mortality for *Bti* was 30 per cent, which was inferior to Ma4 and superior to Vl 8 and Bb 6063.

A high death rate of 83.33 per cent was recorded with Ma4 at 96 HAT which was superior to *Bti* (50 per cent) and Bb5 (53.33 per cent). This was followed by Vl 8 and Bb 6063 with mortality of 16.66 per cent and 13.33 per cent respectively, which were statistically similar. Least mortality was recorded in LsVs 7714 treated insects (6.66 per cent).

At the end of the experiment (5^{th} day) Ma4 recorded 86.66 per cent mortality, which was superior to all other EPF treatments, and was similar to the biocontrol check *Bti* (76.66 per cent). A mortality of 70 per cent was recorded by Bb5, followed by Bb 6063 and Vl 8 (23.33 and 20 per cent, respectively) which was on par with each other. A negligible death rate of 6.66 per cent was recorded by LsVs 7714.

***4.1.2.3 Culex* sp.**

4.1.2.3.1 Larvae

At 24 HAT, highest mortality of 56.66 per cent (Table 5) was observed with Ma4 which was superior to *Bti* (26.66 per cent). Both the isolates of Bb and Vl 8 were found to be similar with mortality of 23.33 per cent each for Bb and 13.33 per cent for Vl 8. LsVs 7714 (3.33 per cent) caused least mortality to the test insects, while complete mortality was recorded in malathion 50 EC treated larvae.

After two days, highest mortality was observed with Ma4 treated larvae (80 per cent). Bb 6063 caused 43.33 per cent mortality which was on par with that of Bb5 (36.66 per cent), Vl 8 (33.33 per cent) and *Bti* (46.66 per cent). Least mortality was observed when treated with LsVs 7714 (6.66 per cent).

At 72 HAT, Ma4 (93.33 per cent mortality) was found to be equally effective as malathion 50 EC, followed by Bb5 (56.66 per cent). The mortality caused by Bb 6063, Vl 8 and *Bti* were on par with each other (40, 33.33 and 53.33 per cent, respectively). LsVs 7714 resulted in lowest mortality of 6.66 per cent.

Table 5. Efficacy of entomopathogenic fungi to *Culex* sp. larvae

Treatments	Cumulative mortality of 4th instar larvae (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	56.66^{b} (48.84)	80.00^{b} (64.63)	93.33^{a} (77.61)	100^{a} (89.71)	100^{a} (89.714)
Bb 5 @ 10^8 spores mL^{-1}	23.33^{cd} (28.78)	36.66^{c} (37.14)	56.66^{b} (48.84)	60.00^{b} (50.76)	66.66^{bc} (54.99)
Bb 6063 @ 10^8 spores mL^{-1}	23.33^{cd} (28.28)	43.33^{c} (41.07)	40.00^{bc} (39.06)	56.66^{b} (48.93)	60.00^{c} (50.85)
Vl 8 @ 10^8 spores mL^{-1}	13.33^{d} (21.14)	33.33^{c} (35.21)	33.33^{bc} (34.22)	56.66^{b} (48.84)	73.33^{b} (59.00)
LsVs 7714 @ 10^8 spores mL^{-1}	3.33^{e} (6.33)	6.66^{d} (12.38)	6.66^{d} (14.62)	13.33^{c} (21.14)	13.33^{d} (21.14)
Bti 5% – biocontrol check	26.66^{c} (30.99)	46.66^{c} (43.07)	53.33^{bc} (46.92)	66.66^{b} (54.99)	70.00^{bc} (56.99)
Malathion 50 EC 0.1% – chemical check	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.71)	100^{a} (89.714)
Untreated control	0^{e} (0.28)	0^{d} (0.28)	0^{d} (0.28)	0^{d} (0.28)	0^{e} (0.286)
CD (0.05)	(9.582)	(12.784)	(16.34)	(7.890)	(7.864)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

Complete mortality was recorded with Ma4 at 96 HAT. Treatment with Bb5 (60 per cent), Bb 6063 (56.66 per cent) and Vl 8 (56.66 per cent) was on par with *Bti* (66.66 per cent). LsVs 7714 recorded least mortality of 13.33 per cent.

At the end of the experimental period (5^{th} day), Vl 8 exhibited a mortality of 73.33 per cent which was similar to that of Bb5 and *Bti* (66.66 and 70 per cent). Bb 6063 exhibited 60 per cent mortality, whereas LsVs 7714 was less effective and no further mortality occurred.

None of the untreated insects died during the experimental period.

4.1.2.3.2 Adults

The mortality of adults treated with different EPF is represented in Table 6. It shows that Ma4 caused 16.66 per cent mortality at 24 HAT, which was statistically similar to *Bti* formulation (10 per cent). Remaining fungi were found to cause only negligible death (3.33 per cent for Bb5) or no death at all (Bb6063, Vl 8, LsVs 7714). Complete mortality was recorded in malathion 50 EC treated test insects.

At 48 HAT, 33.33 per cent death was recorded with Ma4, which was significantly similar to *Bti* (20 per cent), followed by Bb5 (16.66 per cent). Mortality recorded in Vl 8 was significantly lower (13.33 per cent). Bb 6063 and LsVs 7714 caused insignificant death rates of 6.66 per cent and 3.33 per cent.

At 72 HAT, highest mortality was observed in Ma4 (70 per cent), followed by Bb5 (40 per cent) which was similar to *Bti* (50 per cent). Treatment with Bb 6063 and Vl 8 was found to be on par with each other with 13.33 per cent and 23.33 per cent mortality, respectively. A negligible rate of 3.33 per cent mortality was observed in LsVs 7714 treated test insects.

At 96 HAT, effect of Ma4 was equivalent to malathion 50 EC and was found to be superior (86.66 per cent mortality) to Bb5 (63.33 per cent) which was on par with the *Bti* (66.66 per cent). Mortality caused by Vl 8 was 30 per cent which was similar to Bb 6063 (23.33 per cent). Significantly low death rate was recorded with LsVs 7714 with 10 per cent mortality.

Table 6. Efficacy of entomopathogenic fungi to adult *Culex* sp.

Treatments	Cumulative mortality (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma 4 @ 10^8 spores mL^{-1}	16.66^{b} (23.86)	33.33^{b} (35.22)	70.00^{b} (57.00)	86.66^{a} (72.48)	96.66^{a} (83.25)
Bb 5 @ 10^8 spores mL^{-1}	3.33^{cd} (6.75)	16.66^{c} (23.86)	40.00^{c} (39.15)	63.33^{b} (53.07)	76.66^{b} (61.22)
Bb 6063 @ 10^8 spores mL^{-1}	0^{d} (0.91)	6.66^{de} (12.60)	13.33^{d} (21.15)	23.33^{cd} (28.08)	36.66^{c} (36.93)
Vl 8 @ 10^8 spores mL^{-1}	0^{d} (0.91)	13.33^{cd} (21.15)	23.33^{d} (28.78)	30.00^{c} (33.00)	36.66^{c} (37.23)
LsVs 7714 @ 10^8 spores mL^{-1}	0^{d} (0.91)	3.33^{ef} (6.75)	3.33^{e} (6.749)	10.00^{de} (15.30)	13.33^{d} (21.15)
Bti 5% – biocontrol check	10.00^{bc} (15.30)	20.00^{bc} (26.07)	50.00^{c} (45.00)	66.66^{b} (55.08)	73.33^{b} (59.21)
Malathion 50 EC 0.1% – chemical check	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)
Untreated control	0^{d} (0.91)	0^{f} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{e} (0.91)
CD (0.05)	(10.536)	(10.875)	(9.625)	(11.687)	(10.488)

Mean of three replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

Adults of *Culex* sp. exhibited 96.66 per cent mortality when treated with Ma4 at 120 HAT. More than 75 per cent of mortality was observed in Bb5 (76.66 per cent) treated test insects which was on par with *Bti* (73.33 per cent). Bb 6063 and Vl 8 was on par with each other with a mortality of 36.66 per cent each. Least mortality of 13.33 per cent was recorded when treated with LsVs 7714.

Based on pathogenicity test, Ma4 was proved to be the most effective fungus for mosquitoes as the mortality recorded was highest. Therefore further studies were conducted using this fungus.

4.1.3 Effective Dose of *M. anisopliae*

Effective dose of Ma4 was determined based on the dose - mortality response of fourth instar larvae treated with spore suspension concentrations ranging from 10^6 to 10^{10} spores mL^{-1}.

4.1.3.1 Anopheles

Dose - Mortality response of *Anopheles* larvae to varying spore concentrations of Ma4 is indicated in Table 7.

There was no mortality at all at 12 HAT when treated with 10^6 spores mL^{-1}. The corresponding death rate observed with $10^7$and 10^8 was 12.5 and 20 per cent and were similar. At 10^9 spores mL^{-1} the mortality noted was significantly high (32.50 per cent). Highest mortality (40 per cent) was observed with 10^{10} spores mL^{-1}. The trend was exactly same after 24 h. Lower dose of 10^6 and 10^7 spores mL^{-1} recorded 7.5 per cent and 12.5 per cent respectively, which were on par with each other. Higher concentration of 10^8 spores mL^{-1} recorded 22.5 per cent mortality which was on par with 10^9 spores mL^{-1} (40 per cent). Highest spore load (10^{10} spores mL^{-1}) killed 52.5 per cent of test insects.

At 36 HAT, spore concentration of 10^6 spores ml^{-1} caused 15 per cent mortality which was on par with that of 10^7 spores ml^{-1} (22.5 per cent). 10^8 spores mL^{-1} recorded 37.5 per cent mortality which was inferior to mortality caused by 10^9 spores mL^{-1} (55 per cent). Highest mortality was observed in 10^{10} spores mL^{-1} treated insects (70 per cent). Similar trend was noted for the following 24 h also. At 48 HAT,

Table 7. Dose – mortality response of *Metarhizium anisopliae* to *Anopheles* sp. Larvae

Treatments (Spores mL^{-1})	Cumulative mortality (%)							
	12 HAT	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	0^{d} (0.91)	7.50^{dc} (11.70)	15.00^{d} (22.5)	32.50^{d} (34.72)	42.50^{d} (40.61)	60.00^{c} (50.83)	77.50^{c} (62.15)	85.00^{b} (67.50)
10^7	12.50^{c} (18.12)	12.50^{cd} (18.12)	22.50^{d} (27.86)	37.50^{cd} (37.73)	50.00^{d} (45.00)	62.50^{c} (52.34)	82.50^{c} (65.84)	87.50^{b} (69.53)
10^8	20.00^{bc} (26.19)	22.50^{bc} (27.86)	37.50^{c} (37.66)	45.00^{c} (42.17)	67.50^{c} (55.66)	75.00^{c} (60.11)	90.00^{bc} (73.92)	95.00^{a} (80.33)
10^9	32.50^{ab} (34.56)	40.00^{ab} (39.11)	55.00^{b} (47.88)	67.50^{b} (55.44)	82.50^{b} (65.84)	90.00^{b} (73.92)	92.50^{ab} (78.30)	97.50^{a} (84.71)
10^{10}	40.00^{a} (39.17)	52.50^{a} (46.44)	70.00^{a} (56.95)	77.50^{a} (61.77)	92.50^{a} (75.95)	97.50^{a} (84.71)	100^{a} (89.09)	100^{a} (89.09)
Control	0^{d} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{d} (0.91)	0^{d} (0.91)	0^{c} (0.91)
CD (0.05)	(9.446)	(12.706)	(7.205)	(5.312)	(9.928)	(9.850)	(11.883)	(8.951)

Mean of four replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

mortality of 32.5 per cent was recorded in 10^6 spores mL^{-1} treated insects. A slightly high mortality was recorded in the next high spore concentration of 10^7 spores mL^{-1} causing 37.5 per cent mortality, which was on par to each other. The death rate noted with 10^8 spores mL^{-1} was 45 per cent which was inferior to that occurred with 10^9 spores mL^{-1} (67.5 per cent). Highest mortality of 77.5 per cent was recorded by 10^{10} spores mL^{-1}.

At 60 HAT, lower spore concentration of 10^6 spores mL^{-1} recorded 42.5 per cent mortality and was found to be on par with mortality caused by 10^7 spores mL^{-1} (50 per cent). The next higher concentration of 10^8 spores mL^{-1} caused 67.5 per cent which was inferior to mortality observed in 10^9 spores mL^{-1} (82.5 per cent). Spore concentration of 10^{10} spores mL^{-1} recorded the highest mortality of 92.5 per cent. At 72 HAT, as noted in the previous cases, the dosages 10^6, 10^7 and 10^8 spores mL^{-1} were found to be on par with each other with mortality of 60, 62.5 and 75 per cent, respectively. Mortality observed for 10^9 spores mL^{-1} was 90 per cent. Highest mortality of 97.5 per cent was recorded with 10^{10} treated test insects.

At 84 HAT, the mortality caused by 10^6, 10^7 and 10^8 spores was on par (77.5, 82.5 and 90 per cent respectively). Mortality of 10^9 spores mL^{-1} (92.5 per cent) was found to be on par with that recorded in 10^{10} spores mL^{-1} (100 per cent).

At the end of the experimental period (4^{th} day), lower doses 10^6 and 10^7 spores mL^{-1} recorded mortality of 85 and 87.5 per cent, which were on par to each other. Highest spore dose of 10^{10} spores mL^{-1} was found to be on par with the mortality caused by both 10^8 and 10^9 spores mL^{-1} (95 and 97.5 per cent, respectively).

Dose mortality response study of Ma4 to *Anopheles* larvae revealed that mortality of test insects increased with increasing concentration. At the end of experiment the effect of 10^8, 10^9 and 10^{10} spore mL^{-1} were similar. Hence, 10^8 was determined as the effective dose.

4.1.3.2 Aedes

Dose-mortality response of Ma4 to larvae of *Aedes* is indicated in Table 8.

Table 8. Dose – mortality response of *Metarhizium anisopliae* to *Aedes* sp. larvae

Treatments (Spores mL^{-1})	Cumulative mortality (%)							
	12 HAT	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	0^{d} (0.91)	12.50^{c} (21.15)	37.50^{d} (37.66)	42.50^{d} (40.61)	60.00^{c} (50.83)	75.00^{c} (60.11)	82.50^{c} (65.82)	85.00^{c} (67.50)
10^7	12.50^{c} (18.12)	25.00^{b} (30.70)	47.50^{cd} (43.56)	47.50^{cd} (43.56)	62.50^{c} (52.27)	82.50^{bc} (65.84)	90.00^{bc} (73.92)	92.50^{b} (78.30)
10^8	20.00^{bc} (26.19)	35.00^{ab} (37.23)	50.00^{c} (45.00)	65.00^{bc} (53.84)	77.50^{b} (61.77)	85.00^{bc} (67.50)	90.00^{bc} (73.92)	97.50^{ab} (84.71)
10^9	32.50^{ab} (34.72)	42.50^{a} (39.15)	62.50^{b} (52.34)	72.50^{b} (58.61)	82.50^{b} (65.47)	90.00^{b} (73.915)	97.50^{ab} (84.71)	100^{a} (89.09)
10^{10}	40.00^{a} (39.17)	52.50^{a} (43.08)	82.50^{a} (65.84)	87.50^{a} (72.25)	92.50^{a} (78.30)	97.50^{a} (84.71)	100^{a} (89.09)	100^{a} (89.09)
Control	0^{d} (0.91)	0^{d} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{d} (0.91)	0^{d} (0.91)	0^{d} (0.91)	0^{d} (0.91)
CD (0.05)	(8.896)	(7.923)	(7.317)	(10.656)	(9.123)	(10.180)	(11.541)	(9.879)

Mean of four replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

At 12 HAT, less than 50 per cent mortality was observed in all treatments. No mortality was observed when treated with lower dose of 10^6 spores ml^{-1}, whereas that caused by 10^7 and 10^8 spores mL^{-1} were similar (12.5 and 20 per cent mortality). High spore load of 10^9 recorded 32.5 per cent mortality to the test insects, which was similar to the highest spore load of 10^{10} (40 per cent). At 24 HAT, 10^6 spores mL^{-1} resulted in 12.5 per cent mortality, which was inferior to that observed with 10^7 spores mL^{-1}(25 per cent). The spore doses 10^8, 10^9 and 10^{10} were similar in effects with 35, 42.5 per cent and 52.5 per cent mortality, respectively.

At 36 HAT, lowest mortality (37.5 per cent) was observed in the larvae treated with 10^6 spores mL^{-1}, which was on par with that of 10^7 spores mL^{-1} (47.5 per cent). Mortality of 50 per cent was recorded with 10^8 spores mL^{-1} which was inferior to 10^9 spores mL^{-1} that exhibited 62.5 per cent mortality. Highest dose of 10^{10} exhibited high effectiveness to the larvae, causing 82.5 per cent mortality. Similar trend was followed in the next 24 h too, where the lowest mortality of 42.5 and 47.5 per cent was exhibited by the doses, 10^6 and 10^7 spores mL^{-1}. The corresponding mortality with 10^8 spores mL^{-1} was on par with that of 10^9 spores mL^{-1} (65 and 72.5 per cent). The highest dose 10^{10} spores mL^{-1} resulted in 87.5 per cent mortality.

At 60 HAT, 10^6 and 10^7 spores mL^{-1} exhibited mortality of 60 and 62.5 per cent, which was found to be on par with each other. Mortality at 10^8 and 10^9 spores mL^{-1} was observed as 77.5 and 82.5 per cent, respectively which was also statistically similar to each other. Highest mortality of 92.5 per cent was recorded at highest concentration of 10^{10} spores mL^{-1}. At 72 HAT, similar trend was followed with least mortality of 75 per cent with 10^6 spores mL^{-1}. Mortality at 10^7 spores mL^{-1} was observed as 82.5 per cent and was on par with 10^8 and 10^9 spores mL^{-1} which exhibited mortality of 85 and 90 per cent mortality, respectively. The superior treatment 10^{10} spores mL^{-1} resulted in 97.5 per cent death in treated larvae. At 84 HAT, the doses 10^6, 10^7 and 10^8 spores mL^{-1} resulted in significantly high mortality of 82.5, 90 and 90 per cent each, and were similar. Complete mortality was observed with 10^{10} spores mL^{-1} which was on par with that noted with 10^9 spores mL^{-1} (97.5 per cent).

At the end of the experiment (96 HAT), 10^9 spores mL^{-1} also exhibited complete mortality which was similar to 10^8 spores mL^{-1} (97.5 per cent). Dosage of 10^7 spores mL^{-1} (92.5 per cent) was superior to 10^6 spores mL^{-1} (85 per cent).

Dose mortality response of Ma4 to *Aedes* larvae indicated that as the concentration of the fungus increases, mortality of the larvae also increases. At the end of experiment 10^8, 10^9 and 10^{10} spores mL^{-1} was similar to each other. Therefore, 10^8 was selected as the effective dosage.

4.1.3.3 Culex

Dose mortality response of Ma4 to *Culex* larvae is depicted in Table 9. For the first 12 HAT, 10^6 spores mL^{-1} exhibited least mortality of 12.5 per cent. Spore load of 10^7 spores mL^{-1} exhibited 25 per cent mortality, which was found to be inferior than 10^8 spores mL^{-1} with 35 per cent mortality. 10^9 and 10^{10} spores mL^{-1} recorded 57.5 and 62.5 per cent mortality, which was on par to each other. At 24 HAT, lower doses of 10^6 and 10^7 spores mL^{-1} caused 25 and 35 per cent mortality and were found to be similar to each other. Spore load of 10^8 spores mL^{-1} was found to be superior (52.5 per cent) to 10^7, but inferior to 10^9 and 10^{10} spores mL^{-1}. Mortality of 70 per cent each was exhibited when treated with of 10^9 and 10^{10} spores mL^{-1}.

After 36 HAT, lower doses, 10^6 and 10^7 spores mL^{-1} resulted in 42.5 and 52.5 per cent mortality, which were on par with 10^8 spores mL^{-1} (67.5 per cent). 10^9 spores mL^{-1} (80 per cent) found to be statistically similar with 10^{10} spores mL^{-1} causing 85 per cent mortality of the test insects. At 48 HAT, 10^6 and 10^7 spores mL^{-1} exhibited similar mortality of 60 and 70 per cent, while 10^8 and 10^9 resulted in 80 and 90 per cent mortality which were on par with each other. Highest mortality of 97.5 per cent was observed with 10^{10} spores mL^{-1}.

At 60 HAT onwards, 10^8, 10^9 and 10^{10} spores mL^{-1} were on par to each other till the end of the experiment (92.5, 95 and 100 per cent, respectively). Similarly, 10^6 and 10^7 spores mL^{-1} were on par with 72.5 and 82.5 per cent mortality. From 72 to 96 HAT, exactly similar trend was followed in all concentrations. Spore concentration of 10^6 spores mL^{-1} exhibited 82.5 per cent mortality which was inferior to 10^7 spores mL^{-1} (92.5 per cent). Complete mortality was observed in treatments of 10^9 and 10^{10}

Table 9. Dose – mortality response of *Metarhizium anisopliae* to *Culex* sp. larvae

Treatments (Spores mL^{-1})	Cumulative mortality (%)							
	12 HAT	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	12.50^{d} (20.47)	25.00^{c} (29.89)	42.50^{c} (40.67)	60.00^{d} (50.83)	72.50^{b} (58.45)	82.50^{c} (65.84)	85.00^{c} (67.50)	87.50^{c} (69.53)
10^7	25.00^{c} (29.89)	35.00^{c} (36.22)	52.50^{c} (46.44)	70.00^{cd} (56.95)	82.50^{b} (65.84)	92.50^{b} (75.95)	92.50^{b} (75.95)	95.00^{b} (80.33)
10^8	35.00^{b} (36.22)	52.50^{b} (46.44)	67.50^{bc} (55.50)	80.00^{bc} (64.33)	92.50^{a} (78.30)	97.50^{a} (84.71)	100^{a} (89.09)	100^{a} (89.09)
10^9	57.50^{a} (49.39)	70.00^{a} (56.95)	80.00^{ab} (63.81)	90.00^{ab} (73.92)	95.00^{a} (80.33)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)
10^{10}	62.50^{a} (52.34)	70.00^{a} (56.95)	85.00^{a} (67.50)	97.50^{a} (84.71)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)	100^{a} (89.09)
Control	0^{e} (0.91)	0^{d} (0.91)	0^{e} (0.91)	0^{e} (0.91)	0^{c} (0.91)	0^{d} (0.91)	0^{d} (0.91)	0^{d} (0.91)
CD (0.05)	(6.275)	(6.386)	(7.582)	(11.189)	(11.034)	(8.678)	(6.030)	(6.615)

Mean of four replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

spores mL^{-1} which was also found to be on par with 10^8 spores mL^{-1} (97.5 per cent).

At 84 HAT, spore load of 10^6 and 10^7 exhibited mortality of 85 and 92.5 per cent, respectively. 10^8 spores mL^{-1} also exhibited complete mortality as recorded in case of 10^9 and 10^{10} spores mL^{-1}. At the end of the experiment (96 HAT), lowest spore load of 10^6 spores mL^{-1} also recorded significantly high mortality of 87.5 per cent, which was inferior to 10^7 spores mL^{-1} with 95 per cent death of test insects.

It is inferred that from 60 HAT itself 10^8, 10^9 and 10^{10} spores mL^{-1} were similar to each other, and at the end of experiment all these treatments exhibited complete mortality to the test insects. So 10^8 spores mL^{-1} was selected as the effective dose of *M.anisopliae*

Dose mortality response of Ma4 to *Culex* revealed that the species is highly susceptible to the treatments compared to other species *Anopheles* and *Aedes*, with complete mortality of the test insects recorded at 60 HAT with the highest spore dose of 10^{10} spores mL^{-1} and at 84 HAT, complete mortality of test insects was observed at 10^8 spores mL^{-1}.

4.1.4 Lethal Concentration and Lethal Time

Lethal Doses (LC 50 and LC 90) and Lethal Time (LT_{50} and LT_{90}) calculated using dose - mortality response is given below.

4.14.1 Lethal Concentration

The efficacy of conidia of Ma4 on the fourth instar larvae of *Anopheles, Aedes* and *Culex* expressed in terms of LC_{50} and LC_{90} values calculated on the third day after treatment is depicted in Table 10. For *Anopheles* the LC_{50} value worked out was 3.75 x 10^2 spores mL^{-1}, while LC_{90} value was 6.15 x 10^6. In the case of *Aedes* larvae LC_{50} value was 3.3 x 10^3 spores mL^{-1} on the third day of treatment, whereas the corresponding LC_{90} value was 3.21 x 10^6 spores mL^{-1}. The corresponding values calculated for *Culex* was 3.75 x 10^2 spores mL^{-1} and 6.15 x 10^6 spores mL^{-1}, respectively.

Table 10. Lethal concentration of *Metarhizium anisopliae* to mosquitoes on the third day after treatment

Test insect	LC_{50} (Spores mL^{-1})	95% Confidential limit (spores mL^{-1})		LC_{90} (spores mL^{-1})	95% Confidential limit (spores mL^{-1})	
		Upper	Lower		Upper	Lower
Anopheles	3.75×10^{2}	4.04×10^{5}	3.49×10^{-1}	6.15×10^{6}	6.62×10^{9}	5.7×10^{3}
Aedes	3.3×10^{3}	2.19×10^{6}	4.97	3.21×10^{6}	2.13×10^{11}	4.83×10^{5}
Culex	3.75×10^{2}	4.04×10^{5}	3.49×10^{-1}	6.15×10^{6}	6.62×10^{9}	5.7×10^{3}

4.1.4.2 Lethal Time

LT_{50} and LT_{90} computed for Ma4 against *Anopheles* larvae is presented in Table 11. Spore load of 10^6 spores mL^{-1} took 64.62 h for 50 per cent mortality, whereas it took 99.53 h for 90 per cent death. Spore concentration of 10^7 mL^{-1} recorded LT_{50} and LT_{90} values of 58.43 and 99.7 h respectively. At higher concentration 10^8 spores mL^{-1}, the time taken for 50 and 90 per cent death was 46.72 h and 90.33 h, while with 10^9 spores mL^{-1} it was 30.48 and 73.25 h. The time taken with the highest concentration 10^{10} spores mL^{-1} was very less (21.43 and 55.69 h respectively).

LT_{50} and LT_{90} calculated for Ma4 against *Aedes* larvae is furnished in Table 12. Lowest spore load of 10^6 spores mL^{-1} took 54.48 h for 50 per cent mortality, while it took 93.26 h for obtaining 90 per cent mortality. LT_{50} and LT_{90} for 10^7 spores mL^{-1} was 43.79 and 83.74 h, whereas for 10^8 spores mL^{-1}, it was 37.68 and 79.46 h, respectively. LT_{50} values of 10^9 and 10^{10} were 28.18 h and 18.83 h and the corresponding LT_{90} values were 67.88 and 51.36 h, respectively.

LT_{50} and LT_{90} computed for Ma4 against *Culex* larvae is depicted in Table 13. It was revealed that lowest spore concentration of 10^6 spores mL^{-1} recorded the highest LT_{50} of 44.52 h and LT_{90} of 89.32 h. At a concentration of 10^7 spores mL^{-1}, the LC_{50} and LT_{90} values were 33.39 and 74.59 h, respectively. Spore concentration of 10^8 mL^{-1} recorded LT_{50} and LT_{90} values of 23.21 and 55.90 h, whereas those of 10^9 spores mL^{-1} was 20.18 and 53.26 h. Highest spore concentration of 10^{10} spores mL^{-1} recorded LT_{50} and LT_{90} values of 18.83 and 51.36 h, respectively.

Among the three species, *Culex* represented the least LT_{50} values at all spore concentrations, indicating it as the most susceptible species.

4.1.5 Susceptibility of Different Mosquito Stages to *M. anisopliae*

Susceptibility of different stages of all the three mosquito species to Ma4 is depicted in Table 14.

4.1.5.1 Anopheles

Table 11. Lethal time of *Metarhizium anisopliae* spore suspension to *Anopheles* sp. larvae

Treatments (Spores mL^{-1})	Mortality of *Anopheles* larvae at 12 h interval (%)							
	12 HAT*	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	0	7.50	15.00	32.50	42.50	60.00	77.50	85.00
10^7	12.50	12.50	22.50	37.50	50.00	62.50	82.50	87.50
10^8	20.00	22.50	37.50	45.00	67.50	75.00	90.00	95.00
10^9	32.50	40.00	55.00	67.50	82.50	90.00	92.50	97.50
10^{10}	40.00	52.50	70.00	77.50	92.50	97.50	100	100

Treatments (Spores mL^{-1})	LT_{50} (h)	95% Confidential limit (h)		LT_{90} (h)	95% Confidential limit (h)	
		Upper	Lower		Upper	Lower
10^6	64.62	79.33	49.914	99.53	128.04	71.03
10^7	58.43	74.68	42.193	99.70	132.65	66.76
10^8	46.72	63.91	29.544	90.33	122.02	58.64
10^9	30.48	50.76	10.192	73.25	100.99	45.51
10^{10}	21.43	41.27	1.586	55.69	78.01	33.38

Table 12. Lethal time of *Metarhizium anisopliae* spore suspension to *Aedes* sp. larvae

Treatments (Spores mL^{-1})	Mortality of *Aedes* larvae at 12 h interval (%)							
	12 HAT*	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	0	12.50	37.50	42.50	60.00	75.00	82.50	85.00
10^7	12.50	25.00	47.50	47.50	62.50	82.50	90.00	92.50
10^8	20.00	35.00	50.00	65.00	77.50	85.00	90.00	97.50
10^9	32.50	42.50	62.50	72.50	82.50	90.00	97.50	100
10^{10}	40.00	52.50	82.50	87.50	92.50	97.50	100	100

Treatments (Spores mL^{-1})	LT_{50} (h)	95% Confidential limit (h)		LT_{90} (h)	95% Confidential limit (h)	
		Upper	Lower		Upper	Lower
10^6	54.48	69.87	39.10	93.26	122.38	64.14
10^7	43.79	60.09	27.49	83.74	111.47	56.00
10^8	37.68	55.60	19.76	79.46	107.58	51.33
10^9	28.18	47.89	8.46	67.88	93.33	42.42
10^{10}	18.83	39.09	1.43	51.36	72.92	29.80

Table 13. Lethal time of *Metarhizium anisopliae* spore suspension to *Culex* sp. larvae

Treatments (Spores mL^{-1})	Mortality of *Culex* larvae at 12 h interval (%)							
	12 HAT*	24 HAT	36 HAT	48 HAT	60 HAT	72 HAT	84 HAT	96 HAT
10^6	12.50	25.00	33.50	60.00	72.50	82.50	85.00	87.50
10^7	25.00	35.00	52.50	70.00	82.50	92.50	92.50	95.00
10^8	35.00	52.50	67.50	80.00	92.50	97.50	100	100
10^9	57.50	70.00	80.00	90.00	95.00	100	100	100
10^{10}	62.50	70.00	85.00	97.50	100	100	100	100

Treatments (Spores mL^{-1})	LT_{50} (h)	95% Confidential limit (h)		LT_{90} (h)	95% Confidential limit (h)	
		Upper	Lower		Upper	Lower
10^6	44.52	62.34	26.69	89.32	121.59	57.06
10^7	33.39	52.12	14.67	74.59	101.63	47.53
10^8	23.21	41.46	10.96	55.90	77.58	34.23
10^9	20.18	40.89	7.46	53.26	73.33	32.42
10^{10}	18.83	39.09	1.43	51.36	72.92	29.80

After one day, larval mortality recorded was 26 per cent, which was followed by that in adults and pupae with 12 and 4 per cent mortality respectively, which were statistically similar to each other. On the second day, the mortality exhibited by larvae and adult did not vary significantly (42 and 34 per cent mortality). The corresponding pupal mortality was 20 per cent. Highest mortality of 80 per cent was recorded in larvae on the third day, which was followed by that in adults (60 per cent mortality). Least mortality of 38 per cent was observed in pupae.

At the end of the experiment, (fourth day), larvae and adult were equally susceptible causing 94 and 90 per cent respectively. Pupal mortality of 48 per cent was recorded.

4.1.5.2 Aedes

On the first day, highest death rate was observed in larvae with 28 per cent mortality, which was succeeded by adults (14 per cent). Lowest mortality of 6 per cent was observed in pupal population. On the second day, highest mortality in larvae (54 per cent) was observed, followed by adults and pupae (32 and 24 per cent) which were similar to each other. Larval mortality increased to 86 per cent on the third day, while in adults and pupae the mortality recorded was 64 and 36 per cent, which were statistically dissimilar.

At the end of the experiment (fourth day), death observed in larval and adults populations were on par exhibiting 96 and 88 per cent mortality, which was followed by pupal mortality (44 per cent).

4.1.5.3 Culex

On the first day, 50 per cent mortality was recorded in larvae, which was followed by 18 per cent each in adults and pupae. Second day also, the most susceptible stage was the larval stage (86 per cent mortality). Mortality recorded in pupal population was increased to 50 per cent, representing it as the second susceptible stage, whereas adults recorded 32 per cent mortality only.

Table 14. Susceptibility of different stages of mosquitoes to *Metarhizium anisopliae* @ 10^8 spores mL^{-1}

Species & Stage	Cumulative mortality (%)			
	Day 1	Day 2	Day 3	Day 4
Anopheles				
Larva	26.00[a] (30.42)	42.00[a] (40.33)	80.00[a] (64.02)	94.00[a] (78.57)
Pupa	4.00[b] (7.91)	20.00[b] (26.26)	38.00[c] (37.97)	48.00[b] (43.84)
Adult	12.00[b] (18.18)	34.00[a] (35.44)	60.00[b] (50.86)	90.00[a] (73.44)
CD (0.05)	(12.183)	(7.967)	(8.492)	(11.375)
Aedes				
Larva	28.00[a] (31.75)	54.00[a] (47.30)	86.00[a] (68.31)	96.00[a] (82.08)
Pupa	6.00[c] (11.42)	24.00[b] (29.22)	36.00[c] (36.64)	44.00[b] (41.53)
Adult	14.00[b] (21.68)	32.00[b] (34.29)	64.00[b] (53.35)	88.00[a] (71.81)
CD (0.05)	(9.432)	(5.679)	(8.648)	(11.584)
Culex				
Larva	50.00[a] (45.00)	86.00[a] (68.31)	98.00[a] (85.58)	98.00[a] (85.58)
Pupa	18.00[b] (24.64)	50.00[b] (45.00)	50.00[c] (45.00)	54.00[c] (47.30)
Adult	18.00[b] (24.93)	32.00[c] (34.29)	84.00[b] (66.68)	92.00[b] (74.47)
CD (0.05)	(6.625)	(7.153)	(8.571)	(10.682)

Mean of five replications, Figures in the parenthesis are values after angular transformation

No further pupal mortality was observed on the third day, while adults recorded 84 per cent mortality. Mortality observed in larval stage was high (98 per cent).

At the end of the experiment (fourth day), highest mortality was recorded in larvae (98 per cent), which was followed by adults (92 per cent) and pupae (54 per cent).

4.2 DEVELOPENT OF WATER DISPERSIBLE TABLETS FOR THE MANAGEMENT OF MOSQUITO LARVAE

From the above experiments, the most effective entomopathogenic fungus for mosquito control was found to be *M. anisopliae*. The most susceptible mosquito species was *Culex* Therefore, further studies on development of Water Dispersible Tablet (WDT) was carried out using Ma4 which was tested on *Culex* larvae.

4.2.1 Carrier Material

Carrier material was selected based on its effect on germination of the fungal conidia and also by testing the mortality of *Culex* larvae.

4.2.1.1 Effect of carrier materials on germination of M. anisopliae

The results indicating germination percentage in different carrier materials is indicated in Table 15.

`One week after treatment (WAT), the germination percentage did not vary in different carrier materials tested. It was 99.83 per cent in talc, and 99.24 per cent in bran. Complete germination was observed in talc + chitosan (90: 10), while in bran + chitosan (90:10), it was 99.58 per cent.

After two weeks, highest germination was noticed with talc + chitosan (96.16 per cent), which was followed by talc (93.24 per cent). Germination in bran and bran + chitosan was on par to each other with 83.58 and 84.16 per cent, respectively.

Talc + chitosan and talc were found to be equally effective at 3 WAT, their germination percentage being 87.99 and 85.33. Bran + chitosan and bran were also on par with 72.74 and 71.16 per cent germination, respectively.

Table 15. Effect of carrier materials on germination of *Metarhizium anisopliae*

Carrier material	Conidial germination at weekly intervals (%)				
	1st week	2nd week	3rd week	4th week	5th week
Talc	99.83 (88.70)	93.24[b] (75.04)	85.33[a] (67.61)	71.66[a] (57.85)	66.33[b] (54.54)
Bran	99.24 (85.68)	83.58[c] (66.18)	71.16[b] (57.55)	53.91[b] (47.25)	43.16[c] (41.06)
Talc + chitosan	100 (89.83)	96.16[a] (78.77)	87.99[a] (69.81)	76.66[a] (61.17)	71.16[a] (57.53)
Bran + Chitosan	99.58 (87.30)	84.16[c] (66.63)	72.74[b] (58.57)	58.80[b] (50.09)	47.58[c] (43.61)
CD (0.05)	NS	(3.494)	(4.02)	(3.575)	(2.838)

Mean of four replications, Figures in the parenthesis are values after angular transformation

After four weeks, exactly similar trend was noted in all the treatments. Maximum germination was observed in talc + chitosan (76.66 per cent), which was statistically similar to that in talc (71.66 per cent). Germination observed in bran and bran + chitosan were on par with 53.91 and 58.83 per cent, respectively.

At the end of the experiment (5 WAT), talc + chitosan exhibited highest germination (71.16 per cent) and was found to be superior to all other carrier materials. The next superior treatment was talc (66.33 per cent) followed by that in bran and bran + chitosan (43.16 and 47.58 per cent) which were on par with each other.

4.2.1.2 Effect of carrier materials on mortality of Culex larvae

Effect of different carrier material on mortality of *Culex* larvae is presented in Table 16.

At 24 HAT, the carrier materials did not cause any significant variation in mortality to the test insect. Talc + chitosan and bran + chitosan recorded 10 per cent mortality in each, whereas talc and bran recorded 7.5 per cent mortality in each.

Effect of talc + chitosan and talc was on par on the second day, as they recorded 32.5 and 30 per cent mortality to the larvae. This was followed by bran and bran + chitosan that recorded 15 per cent mortality in each.

On the third day, talc + chitosan recorded 60 per cent mortality, which was on par with that observed in talc (52.5 per cent). Less than 50 per cent mortality was observed in bran and bran + chitosan (40 and 30 per cent, respectively) which was statistically similar.

A similar trend was noticed on 96 HAT, where talc + chitosan and talc exhibited 87.5 and 80 per cent mortality, which were on par. Bran + chitosan and bran recorded 70 and 57.5 per cent, respectively, which were also similar.

At the end of the experiment (fifth day), talc + chitosan was observed as the superior treatment with 95 per cent mortality, which was succeeded by talc (87.5 per cent). Bran + chitosan resulted in 80 per cent mortality which was similar to that in talc. The least effective carrier was bran causing only 70 per cent mortality.

Table 16. Effect of carrier materials on virulence of *Metarhizium anisopliae* to *Culex* sp. larvae

Carrier material	Cumulative mortality (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Talc	7.50^{a} (11.70)	30.00^{a} (33.05)	52.50^{ab} (46.44)	80.00^{ab} (63.80)	87.50^{b} (69.53)
Bran	7.50^{a} (14.05)	15.00^{b} (22.50)	40.00^{bc} (39.16)	57.50^{c} (56.94)	70.00^{c} (56.94)
Talc + chitosan	10.00^{a} (16.08)	32.50^{a} (34.34)	60.00^{a} (50.83)	87.50^{a} (69.53)	95.00^{a} (80.33)
Bran + Chitosan	10.00^{a} (16.08)	15.00^{b} (22.50)	30.00^{c} (33.05)	70.00^{bc} (56.94)	80.00^{bc} (63.80)
Control	0^{b} (0.28)	0^{c} (0.28)	0^{d} (0.28)	0^{d} (0.28)	0^{d} (0.28)
CD (0.05)	(7.563)	(9.653)	(7.842)	(8.138)	(10.401)

Mean of four replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

Talc + chitosan was observed as the superior carrier material, as it recorded both highest spore germination and mortality of larvae. Therefore, further studies were carried out using this carrier material.

4.2.2 Binding Agents

The effect of binding agents on viability and virulence of Ma4 formulated in talc + chitosan is presented below

4.2.2.1 Viability

Viability in terms of germination percentage of Ma4 conidia as influenced by the binding agents is depicted in Table 17.

After one week, there was no statistical difference in conidial germination among the treatments. There was complete germination in Carboxy Methyl Cellulose 7% (CMC), Acacia Gum arabic 5% (AG), Microcrystalline Cellulose 7% (MCC), whereas 99.41 per cent was recorded in Polyvinyl Pyrrolidone 5% (PVP). Germination observed in the control was 99.58 per cent. Two weeks after treatment, highest germination was recorded when CMC was used as binding agent (98.66 per cent), which was followed by MCC (92.08 per cent) which was on par with control (89.91 per cent). It was noticed that the germination in AG (80.99 per cent) and PVP (74.33 per cent) were also similar to each other and inferior to other treatments.

Conidial germination was highest in CMC (87.83 per cent) after three weeks of treatment, followed by that in MCC (80.32 per cent), which in turn was statistically similar to control (77.41 per cent). Germination in AG was 62.41 per cent which was superior to PVP (50.16 per cent). CMC recorded highest germination after fourth week (72.08 per cent) which was on par with that of MCC (67.07 per cent) and control (69.08 per cent). Conidial germination observed was less when AG (43.58 per cent) and PVP (39.57 per cent) were added.

At the end of the experimental period (fifth week), CMC (62.66 per cent germination) was noted as the superior binder followed by MCC (53.82 per cent) and control (51.413 per cent). It was noted that the conidial germination drastically

Table 17. Effect of binding agents on germination of *Metarhizium anisopliae*

Treatments	Conidial germination at weekly intervals (%)				
	1st week	2nd week	3rd week	4th week	5th week
Ma4 (10^{10} spores mL^{-1}) + talc + chitosan (90:10) + CMC 7%	100 (89.835)	98.66[a] (84.24)	87.83[a] (69.64)	72.08[a] (58.11)	62.66[a] (52.34)
Ma4 (10^{10} spores mL^{-1}) + talc + chitosan (90:10) + PVP 5%	99.41 (87.67)	74.33[c] (59.62)	50.16[d] (45.09)	39.57[b] (38.98)	29.24[c] (32.70)
Ma4 (10^{10} spores mL^{-1}) + talc + chitosan (90:10) + AG 5%	100 (89.83)	80.99[c] (64.20)	62.41[c] (52.19)	43.58[b] (41.29)	32.49[c] (34.71)
Ma4 (10^{10} spores mL^{-1}) + talc + chitosan (90:10) + MCC 7%	100 (89.83)	92.08[b] (74.12)	80.32[b] (63.74)	67.07[a] (55.01)	53.82[b] (47.20)
Control (Without binding agent)	99.58 (88.02)	89.91[b] (71.58)	77.41[b] (61.65)	69.08[a] (56.24)	51.41[b] (45.81)
CD (0.05)	NS	(5.074)	(3.458)	(3.120)	(3.389)

Mean of four replications, Figures in the parenthesis are values after angular transformation

declined in treatments where AG (32.49 per cent) and PVP (29.24 per cent) were used.

4.2.2.2 Virulence to Culex larvae

Effect of binding agents on virulence of Ma4 tested against *Culex* larvae is depicted in Table 18. There was no variation among treatments after 24 h, mortality of test insects ranging from 5 to 12.5 per cent.

On the second day, the effect of CMC and MCC was to be found similar causing 35 and 32.5 per cent mortality respectively. Mortality noted in AG (27.5 per cent) was on par with that in control (22.5 per cent). Least mortality of 15 per cent was observed in PVP.

MCC and CMC were equal in effect and superior to other treatments at 72 HAT, with highest mortality of 67.5 and 65 per cent, while with AG it was 47.5 per cent, which was on par with control (50 per cent). PVP recorded least mortality of 32.5 per cent. At 96 HAT, CMC and MCC showed high mortality that was on par with that of control, the values being 85, 82.5 and 77.5 per cent, respectively. Mortality with the addition of AG and PVP were significantly less (62.5 and 57.5 per cent), which were statistically similar.

At the end of experiment (fifth day), CMC that caused 95 per cent mortality was the superior. The next superior treatment was MCC that exhibited 90 per cent mortality, which was on par with control (85 per cent). AG and PVP recorded 77.5 and 70 per cent, mortality respectively.

The results revealed that CMC was the superior material that can be used as a binding agent, without affecting the viability or virulence of *M. anisopliae*. Therefore, Ma4 tablets formulated with the carrier, talc + chitosan (90:10) and the binding agent CMC 7 % was further assessed to determine the ideal moisture content and shelf life.

4.3 SHELF LIFE OF TABLETS AT VARYING MOISTURE LEVELS

Shelf life of tablets stored at ambient conditions, assessed based on their viability and virulence at varying moisture contents of 8, 10 and 15 % is presented below.

Table 18. Effect of binding agents on virulence of *Metarhizium anisopliae* to *Culex* sp. larvae

Treatments	Cumulative mortality (%)				
	24 HAT	48 HAT	72 HAT	96 HAT	120 HAT
Ma4 (10^{10} spores mL^{-1}) + talc+ chitosan (90:10) +CMC 7%	10.00^{a} (16.08)	35.00^{a} (36.00)	65.00^{a} (53.99)	85.00^{a} (67.50)	95.00^{a} (80.33)
Ma4 (10^{10} spores mL^{-1}) + talc+ chitosan (90:10) +PVP 5%	5.00^{a} (9.67)	15.00^{b} (22.50)	32.50^{c} (34.71)	57.50^{c} (49.38)	70.00^{d} (56.94)
Ma4 (10^{10} spores mL^{-1}) + talc+ chitosan (90:10) +AG 5%	7.50^{a} (14.05)	27.50^{ab} (34.54)	47.50^{b} (43.55)	62.50^{bc} (52.55)	77.50^{cd} (61.77)
Ma4 (10^{10} spores mL^{-1}) + talc+ chitosan (90:10) + MCC 7%	12.50^{a} (20.46)	32.50^{a} (34.56)	67.50^{a} (55.44)	82.50^{a} (65.83)	90.00^{b} (71.56)
Control (Without binding agent)	7.50^{a} (11.70)	22.50^{ab} (27.85)	50.00^{b} (45.00)	77.50^{ab} (61.77)	85.00^{bc} (67.50)
Untreated control	0^{b} (0.28)	0^{c} (0.28)	0^{d} (0.28)	0^{d} (0.28)	0^{e} (0.28)
CD (0.05)	(10.28)	(9.08)	(8.549)	(9.515)	(8.584)

Mean of four replications, HAT - Hours After Treatment, Figures in the parenthesis are values after angular transformation

4.3.1 Viability

Germination of Ma4 tablets at varying moisture content is depicted in Table 19.

After two weeks, tablets formulated with 15% moisture recorded highest germination (98.72 per cent), which was similar with those tabulated with 10 % (97.26 per cent). Least germination was observed in 8 % tablets (95.59 per cent). At the end of the first month, same trend was followed with statistically similar values at 15 and 10 per cent moisture levels (89.66 and 87.86 per cent), which was followed by the germination percentage at 8% moisture (79.66 per cent).

The germination percentage was 78.66 at 15 %, 71.79 at 10% and 58.73 per cent at 8% moisture and were dissimilar at six WAS, while it gradually reduced to 65.06, 56.93 and 44.46 per cent for tablets with 15, 10 and 8% moisture, 8 WAS , which were also dissimilar to each other. At 10 WAS, tablets with highest germination was observed in 15 % moisture (52.26 per cent), which was followed by 10 % and 8 % with 40.59 and 27.66 per cent germination, respectively.

At the end of the experimental period, the germination rate of 15 % (33.66 per cent), and 10 % cent moisture (30.66 per cent), was on par. Least germination of 17.19 per cent, observed in tablets formulated at 8 % moisture.

4.3.2 Virulence to *Culex* Larvae

Virulence of Ma4 tablets formulated under varying moisture levels, assessed based on mortality caused to *Culex* larvae on the third day after treatment is depicted in Table 20.

After one month storage, highest mortality was recorded in larvae treated with tablets of 15% moisture content (96 per cent), followed by that of 10% and 8% (80 and 74 per cent mortality). After two months the mortality of larvae did not vary with moisture content of tablets. It was 88, 78 and 68 with 15, 10 and 8% respectively. At the end of the experimental period (third months), significantly high mortality of 72 per cent was observed when treated with tablets having 15% moisture, while the

Table 19. Shelf life of *Metarhizium anisopliae* tablets at varying moisture levels - Viability

Weeks After Storage	Germination of *M. anisopliae* tablets (%) at varying moisture levels			
	8 %	10 %	15 %	CD (0.05)
2	95.59[b] (78.04)	97.26[ab] (80.84)	98.72[a] (83.94)	(3.612)
4	79.66[b] (63.25)	87.86[a] (69.67)	89.66[a] (71.38)	(3.304)
6	58.73[c] (50.03)	71.79[b] (57.93)	78.66[a] (62.50)	(1.886)
8	44.46[c] (41.80)	56.93[b] (48.99)	65.06[a] (53.78)	(3.3)
10	27.66[c] (31.70)	40.59[b] (39.56)	52.26[a] (46.29)	(3.048)
12	17.19[b] (24.42)	30.66[a] (33.60)	33.66[a] (35.45)	(2.74)

Mean of five replications, Figures in parenthesis are values after angular transformation

Table 20. Shelf life of *Metarhizium anisopliae* tablets at varying moisture levels - Virulence

Months After Storage	Cumulative mortality of *Culex* larvae on the third day (%) at varying moisture levels				
	8 %	10 %	15 %	Control	CD (0.05)
1	74.00[b] (59.86)	80.00[b] (63.73)	96.00[a] (82.08)	0[c] (0.91)	(9.056)
2	68.00[b] (5.71)	78.00[ab] (63.40)	88.00[a] (72.11)	0[c] (0.91)	(9.386)
3	46.00[b] (42.69)	52.00[b] (46.15)	72.00[a] (58.66)	0[c] (0.91)	(9.451)

Mean of five replications, Figures in parenthesis are values after angular transformation

larvae treated with tablets of 10 and 8 % per cent did not differ significantly (52 and 46 per cent respectively).

4.3.3 Extent of Contamination in tablets

Tablets (10^{10} spores mL^{-1}), when examined at 15 DAS exhibited certain contaminants such as *Aspergillus* and *Penicillium* (Plate 18). The extent of contamination noticed in tablets of 8 and 10% moisture did not vary significantly, though the contaminants are more in 15%, the cfu values being 4×10^4 mL^{-1}, 6×10^4 mL^{-1} and 1.2×10^5 mL^{-1}, respectively (Table 21).

4.3.4 Physical Properties

Tablets formulated were circular in shape with 1cm thickness and 1.5 cm diameter. Disintegration time noted was 8.20 min and the friability was 1.5%.

4.4 BIOEFFICACY OF TABLETS IN MANAGING LARVAE

Bioefficacy of *M.anisopliae* tablets tested on larvae of *Culex* under laboratory as well as field conditions is presented below.

4.4.1 Laboratory Conditions

The data on efficacy of Ma4 tablets on larval mortality and adult emergence of *Culex* larvae is presented in Table 22.

On the third day of treatment, highest mortality was exhibited when treated with 4 tablets (40.75 per cent) which was found to be similar to mortality recorded in 3 tablets (37.25 per cent). Treatment with 1 and 2 tablets resulted 29.5 and 30.25 per cent mortality, which was on par with each other. Mortality recorded in control was 4.75 per cent.

Highest mortality of 71.25 per cent was observed on the sixth day, with 4 tablets. Mortality caused by 1, 2 and 3 tablets was statistically similar, values being 67.5, 62.25 and 59.5 per cent mortality. In the control, 11 per cent mortality was recorded.

On the ninth day of treatment, highest mortality was observed when treated with 4 tablets, that caused 83 per cent mortality, while to that of 3 tablets was 82 per

Table 21. Extent of contamination in *Metarhizium anisopliae* tablets

Moisture content (%)	No. of cfu (10^5 mL^{-1}) at 15 DAS
8	0.40^{ab} (0.91)
10	0.60^{ab} (1.01)
15	1.20^{a} (1.26)
Control (Spore suspension)	0^{b} (0.70)
CD (0.05)	(0.361)

Mean of five replications, DAS - Days After Storage, Figures in parenthesis are values after square root transformation.

18a. 8% moisture

18b. 10% moisture

18c. 15% moisture

18d. Spore suspension

Plate 18. Extent of contamination in *Metarhizium anisopliae* tablets

Table 22. Bioefficacy of *Metarhizium anisopliae* tablets on *Culex* larvae under laboratory conditions

No. of tablets L-1	Cumulative larval mortality (%)			Adult emergence (%)
	3 DAT	6 DAT	9 DAT	
1	29.50^{c} (32.80)	62.25^{ab} (52.12)	74.25^{b} (59.56)	25.50^{b} (30.26)
2	30.25^{bc} (33.32)	59.50^{b} (50.51)	74.50^{b} (59.82)	25.00^{b} (29.82)
3	37.25^{ab} (37.58)	67.50^{ab} (55.36)	82.00^{a} (65.00)	17.00^{c} (24.29)
4	40.75^{a} (39.63)	71.25^{a} (57.68)	83.00^{a} (65.72)	16.00^{c} (23.42)
Control	4.75^{d} (12.443)	11^{c} (19.33)	18.25^{c} (25.565)	81.00^{a} (64.56)
CD (0.05)	(4.697)	(5.565)	(4.597)	(4.922)

Mean of four replications, DAT - Days After Treatment, Figures in parenthesis are values after angular transformation

cent, which were statistically similar. At the same time, treatments of 1 and 2 tablets were also similar causing mortality of 74.25 and 74.5 per cent, respectively. Mortality of 18.25 per cent was recorded in control.

The adult emergence from treated larvae indicated the efficacy of tablets in managing mosquitoes. As the dosage increased from 1 to 4, the rate of emergence was found to decrease. The emergence rate noted was significantly lower when treated with the higher doses of 4 and 3 tablets L^{-1}. The rate of emergence noted was 17 and 16 respectively. When the dosage was reduced to 2 and 1 the corresponding emergence was 25 and 25.5 which were not dissimilar, while in untreated control there was 81 per cent emergence.

4.4.2 Field Conditions

Efficacy of tablets under field conditions is presented in Table 23. Larvae collected by natural oviposition under field conditions when treated with tablets @ 10 L^{-1} exhibited mortality that varied with dosage. Four Days After Treatment (DAT), larvae treated with 10 and 9 tablets recorded 37.86 and 36.03 per cent mortality which were on par with each other, which was followed by 8 and 7 tablets which recorded 31.57 and 31.69 per cent mortality . Least mortality of 1.33 per cent was recorded in control.

Seven DAT, mortality noted in the 10 and 9 tablets were similar, values being 48.26 and 44.77 while those of lower doses 8 and 7 tablets were 39.73 and 38.55 per cent, respectively. Only a negligible rate of 2.89 per cent was observed in control. At the end of the experiment (10 DAT), larvae either got pupated or died. More than 70 per cent mortality was observed in all the treatments. A death rate of 84.1 and 83.97 per cent was noted with 10 and 9 tablets, which were not significantly different. Mortality noted with 8 and 7 tablets was on par with each other (79.59 and 75.03 per cent). The corresponding rate in control was 4.37 per cent. Therefore, the dosage for field level was fixed as 9 tablets for 10 L.

Table 23. Mortality of *Culex* larvae treated with *Metarhizium anisopliae* tablets

No. of tablets $10\ L^{-1}$	Cumulative mortality (%)		
	4 DAT	7 DAT	10 DAT
7	31.69^{b} (34.25)	39.73^{b} (39.06)	75.03^{b} (60.07)
8	31.57^{b} (34.18)	38.55^{b} (37.86)	79.59^{b} (63.15)
9	36.03^{a} (36.87)	44.77^{a} (41.99)	83.97^{a} (66.46)
10	37.86^{a} (37.95)	48.26^{a} (43.99)	84.10^{a} (66.61)
Control	1.33^{c} (6.62)	2.89^{c} (9.78)	4.37^{c} (12.06)
CD (0.05)	(2.401)	(3.028)	(3.168)

Mean of four replications, DAT - Days After Treatment, Figures in parenthesis are values after angular transformation

Discussion

5. DISCUSSION

The spread of mosquitoes has resulted in the growth of vector-borne diseases, posing serious public health problems. Overuse of insecticides increase the resistance in mosquitoes leading to negative consequences in the environment and non-target organisms. For decades, scientists have been looking for pathogens that can be employed in mosquito control operations. While an increasing number of studies have been focused on bacteria, other microbial pathogens such as fungi were largely ignored. Fungi such as *Lagenidium, Coelomomyces* and *Culicinomyces* are known to infect mosquitoes, and have been studied extensively by Scholte *et al.* (2004). However, entomopathogenic fungi (EPF) such as *Metarhizium anisopliae* (Metsch.) Sorokin, *Beauveria bassiana* (Bals.) Vuillemin and *Lecanicillium muscarium* Zare and Gams were isolated from mosquitoes, which were found to infect and kill the larvae and adults (Vyas *et al.*, 2007, Luz *et al.*, 2010).

Amenability to mass multiplication, environmental persistence along with the inability of the conidia to germinate in the mosquito habitat until it is actually exposed to a host, makes EPF a promising biocontrol agent (Roberts, 1970). EPF are the most host specific microbial agents that infect mosquitoes, and are known to cause natural infections as well as epizootics (Geetha and Balaraman, 1999). Fungal infection in mosquitoes begin with adherence of conidium to the cuticle and formation of germ tube, breaching of cuticle with mechanical pressure and production of cuticle degrading enzymes as observed in the case of any other insects (Pedrini *et al.*, 2007).

5.1 PATHOGENICITY OF ENTOMOPATHOGENIC FUNGI TO MOSQUITOES

Pathogenicity is a qualitative trait referring to the inherent genetic capacity of a microorganism to cause disease that is mediated by specific virulence factors.

In the present research work, pathogenicity studies of *M. anisopliae* NBAIR isolate Ma4, *B. bassiana* NBAIR isolate Bb5 and KAU isolate Bb 6063, *L. lecanii* NBAIR isolate, Vl 8 and *L. saksenae* KAU isolate 7714 were carried out in the three common mosquito species of Kerala. All the fungi tested were pathogenic to *Anopheles*, *Aedes* and *Culex* at varying levels. Infected larvae exhibited almost similar symptoms of mycosis. Reduced activity at 12 Hours After Treatment (HAT) with

sinking movement, colour change from dark grey to white, presence of copious amount of mucus around the body and gut degeneration were the symptoms of mycosis. Death of the infected larvae was noticed within 24 HAT. Infected adults were inactive and died within 24 h of treatment, followed by mummification. Green sporulation was noticed in *M. anisopliae* treated adults 72 HAT. *L. saksenae* was less infective to mosquitoes.

Gut disintegration observed in this study ay be attributed to the degeneration of tissues, with loss of structural integrity of membranes and dehydration of cells caused by the fungal toxins, as suggested by Ferron (1981). In his study, *M. anisopliae* was reported to cause similar symptoms in the elaterid *Melanotus punctolineatus* Pelerin. Likewise, Farida *et al.* (2018) reported alterations and malformations in the intestine, adipose tissue and haemolymph of *Cx. pipiens* larvae infected by *B. bassiana*.

Kannan *et al.* (2008) reported that the fungal spores supressed the cellular defence system in *An. stephensi* and the mycelial growth on legs and wings arrested the movement in adults. Post death symptoms in adults of *Anopheles, Aedes* and *Culex* caused by *M. anisopliae* observed in this study was similar to those reported by Scholte *et al.* (2007) in *Ae. albopictus* adults infected with *M. anisopliae.* Scholte *et al*. (2008) opined that the *M. anisopliae* spores upon reaching the haemocoel utilizes the insect nutrients and secrete toxins that eventually kill the adults of *An. gambiae*.

Mortality recorded at 24 h interval revealed that, *M. anisopliae* was superior to all other fungi as it exhibited highest mortality (83.33 to 100 per cent), five days after treatment (DAT) to both larvae and adults of all the three mosquito species, followed by *B. bassiana* isolates. *Lecanicillium* spp. were less infective. Its effect was superior to *Bacillus thuringiensis israelensis* Barjac (*Bti*) which is the most commonly used biocontrol agent for mosquito control (73.33 and 66.66 to per cent mortality) and equally effective as the insecticide malathion 50 EC.

These findings clearly states that the NBAIR isolates Ma4 and Bb5 are highly pathogenic to mosquitoes. Perusal of literature reveals that even other isolates of *M. anisopliae* and *B. bassiana* are effective to mosquitoes.

Scholte *et al.* (2007) observed that the strain ICIPE-30 of *M.anisopliae* exhibited 89.3 ± 2.2 and 87.1 ± 2.65 per cent mortality in *Ae. albopictis* and *Ae. aegypti,* respectively. Choi *et al*. (2020) stated that the isolates CN6S1W1 was the most effective among the 65 isolates screened against mosquitoes, as it resulted in 90.5 ± 9.5 and 85 ± 12.2 per cent mortality in *Ae. albopictus* and *Cx. pipiens* adults, five DAT.

Comparitive studies on the efficacy of *M. anisopliae* and *B. bassiana* to different species of mosquitoes were undertaken by various researchers, worldwide. The findings of de paula *et al.* (2008) were in parity with present study as they reported the superiority of *M. anisopliae* LPP133 over *B. bassiana* CG494 in managing *Ae. aegypti*. The adult mortality recorded was 89.33 per cent in *M. anisolpiae* while it was only 70.67 per cent mortality in *B. bassiana*. Bukhari *et al.* (2011), while attempting the utilization of shellsol oil formulation of *B. bassiana* and *M. anisopliae*, reported that both the fungi were equally effective in managing *An. gambiae* population, as the pupation was reduced by 39 to 50 per cent. Vivekanandan *et al*. (2020a) while working with the isolate VKKH3 of *M. aniopliae* and VKBb03 of *B. bassiana* reported lesser death rate of 45.33 and 2.66 per cent in *Ae. aegypti,* while it was and 41.33 and zero per cent in *Cx. quinquefasciatus* on the 4th DAT. *L. lecanii* isolate VKPH1 was not infective. Their observations are in concurrence with the present study, as in both these experiments, *M. anisopliae* was the most effective fungus and *Lecanicillium* was the least effective one.

The infectivity of *B. bassiana* observed in this study, to the tune of 56.66 to 83.33 per cent in adults and larvae of *Anopheles*, *Aedes* and *Culex* is substantiated by the findings of several researchers. In a comparative study of two different strains of *B. bassiana*, CG 24 and CG 494, Pereira *et al.* (2009) observed that CG 24 was highly virulent against *Ae. aegypti* larvae, recording 82.7 ± 23.9 per cent mortality, while in the other it was only 6 ± 0.5 per cent. Variation in virulence even within the species is a common phenomenon among entomopathogens.

Hamid *et al.* (2013) recorded 100 per cent mortality of *Cx. pipiens* larvae on the fifth DAT, when treated with *B. bassiana* representing high virulence of the strain.

Lee *et al.* (2019) reported that the strain JNR1W1 recorded 72.3 ± 10.1 and 66.2 ± 4.5 per cent death in *Ae. albopictis* and *Cx. pipiens* adults.

Bti, biocontrol check in this study (green milestone @ 5%) resulted in 66.66 to 73.33 per cent mortality in larvae and 66.66 to 76.66 per cent mortality in adults on the fifth DAT. The results also revealed that the effect of *Bti* was inferior to *M. anisopliae* that caused 100 per cent larval mortality, but on par with *B. bassiana* that caused 83.33 per cent death.

On the other hand, in the studies conducted by Fernando (1993) it was observed that *Bti* was superior to *M. anisopliae*, and the combination of *Bti* with *M. anisopliae* had a synergistic effect on larvae of *Anopheles punctipennis* Say. When *M. anisopliae* alone was treated as dry conidia @ 7.5 mg 80 mL^{-1} there was no mortality till 12 HAT, with *Bti* (bactimos WP) @ 2.12 mg 80 mL^{-1} there was 37.5 per cent mortality while their combination resulted in 62.5 per cent mortality.

The chemical check fixed in this study was the contact insecticide malathion 50 EC 0.1% that is recommended by CIB & RC for mosquito control programmes in the country. This study confirmed its efficacy in controlling mosquitoes, as it resulted in 100 per cent death of larvae and adults, within 24 HAT. Efficiency of malathion to cause 100 per cent mortality to *Ae. aegypti* and *Ae. albopictus* adults was earlier reported by Xue (2008) and to *An. gambiae* and *Cx. quinquefasciaus* by Fagbohun *et al.* (2020).

Although chemical insecticides are effective in controlling mosquito larvae in water bodies, its negative impact on environment had been cited in various studies. Stahl, (2000) pointed out the ill effects of spraying malathion 50 EC and permethrin 5 EC in stagnant water for the management of mosquitoes. Acute and chronic health hazards such as skin and eye irritations, headache, dizziness, difficulty in breathing, physiological and immune system disorders were some of the ill effects reported in human beings. Furthermore, death of several non target organisms such as fishes, shrimps, mussels etc. were reported by Miliam *et al.* (2000) due to the application of organophosphates and synthetic pyrethroids in water bodies. All the more, resistance to various pesticides developed by mosquitoes have led to the resurgence of mosquito

borne diseases (Dahmana and Mediannikov, 2020). All these adversities can be mitigated by the use of microbials that are comparatively safer and environment friendly due to their specificity and less chances of resistance build up (Hegazy *et al.,* 2021).

Dose-mortality studies of *M. anisopliae* carried in the 4th instar larvae using varying concentrations ranging from 10^6 to 10^{10} spores mL^{-1}, revealed that the rate of death increased with the increase in concentration of spores. As the effect of 10^8 spores mL^{-1} was equivalent to 10^9 and 10^{10} spores mL^{-1}, 10^8 spores mL^{-1} was fixed as the effective dose, to save the inoculum. Mortality recorded was 95 to 100 per cent in all the three species on the 4th DAT. LC_{90} values computed on the third DAT for *Anopheles, Aedes* and *Culex* was 10^6 spores mL^{-1}.

The findings of Benserradj and Mihoubi (2014) regarding the dose - mortality response of an Algerian isolate of *M. anisopliae* in the 4th instar larvae of *Cx. pipiens* was in parity with the findings of the present study. The study reported that as the concentration increased from 10^5 to 10^9 spores mL^{-1}, mortality also increased and that at a higher dose of 10^9 spores mL^{-1}, there was 96 per cent mortality on the 4th DAT. However, the LC_{50} and LC_{90} values recorded at 72 HAT were higher in their studies. The LC_{50} was 10^6 spores mL^{-1} with the Algerian isolate, while it was 10^2 spores mL^{-1} in the present investigation with NBAIR isolate Ma4, clearly disclosing the superiority of Ma4 in mosquito control.

The experiments carried out by Geetha and Balaraman (1999) and Vivekanandan *et al.* (2020a) also revealed a dose dependant virulence of entomopathogenic fungi such as *M. anisopliae* and *B.bassiana* on *An. stephensi, Ae. aegypti* and *Cx. quinquefasciatus*.

LT_{90} values of *M. anisopliae* @ 10^8 spores mL^{-1} calculated in this investigation, on *Anoplheles, Aedes* and *Culex* revealed that, *Culex* was more susceptible, followed by *Aedes* and *Anopheles* (55.9 h, 79.46 h and 90.33 h, respectively). On the contrary, Vivekanandan *et al.* (2020a), reported that *An. stephensi* and *Ae. aegypti* were more susceptible to *M. anisopliae* strain VKKH3 @ 10^{10} spores mL^{-1} than *Cx. quinquefasciatus.* The LT_{90} values were 132.24 h, 122.64

h and 145.92 h respectively. They attributed the increased susceptibility of *An. stephensi* to its surface feeding behaviour, unlike that of the other two species.

Present investigation revealed the susceptibility of larval stage, while pupal stage was the least susceptible, irrespective of the species. Carlino *et al.* (2019) reported that *Ae. aegypti* pupae were more susceptible to the blastospores of *M. anisopliae* (10^7 spores mL^{-1}) than the conidia as the former resulted in complete mortality, 24 HAT while the latter exhibited only 43.5 per cent mortality. The less susceptibility of pupae noted in this study might be due to the fact that the spore suspension used was more of aerial conidia and less of blastospores as the mass production method adopted was static liquid fermentation.

5.2 DEVELOPMENT OF WATER DISPERSIBLE TABLETS

Tablets are compressed mass of active ingredient along with other additives having usually a circular shape (FAO, 2010). Compressed formulations minimise the risk of dustiness and contamination. Water dispersible nature of the tablets enables easy disintegration in water. In addition to these desirable characteristics, microbial formulations should be viable and virulent during storage.

Keeping these aspects in view, a protocol for formulating water dispersible compressed tablets was developed by standardising a suitable carrier material, binding agent and ideal moisture content that could maintain the viability and virulence of the fungus for a period of three months of storage.

The first and foremost step was to standardise a suitable carrier material. A good carrier material should be non-toxic, easy to process, free of lump formation, cheap and easily available (Somasegaran and Hoben, 1994).

Experiment to standardize a suitable carrier material to formulate *M. anisopliae* tablets, revealed the superiority of talc + chitosan in the ratio 90:10 as there was 71.16 per cent conidial germination and 95 per cent larval mortality in *Culex* larvae on the 5^{th} week after storage (WAS), compared to the carriers talc, bran or bran + chitosan (90:10). The corresponding viability and virulence in talc was

66.33 and 87.5 per cent, while in bran it was 43.16 and 70 per cent and in bran + chitosan, it was 47.58 and 80 per cent, respectively.

Such research works on development of smart formulations like tablets and capsules based on EPF is meagre, except for a few attempts which were not for mosquito control.

Superiority of chitosan, as a carrier for EPF formulations as observed in this study was earlier stated by Remya (2018). In the gel formulation of *M.anisopliae* and *B.bassiana* developed by Remya (2018), chitosan based Metarhizium gels had better viability (2.19 x 10^6 mL^{-1} cfu) when compared to alginate and gelatin based gels (1.86 x 10^6 and 1.03 x 10^6 mL^{-1} cfu), even after 75 days of storage. Remya and Reji (2020) observed that chitosan as an ideal carrier for capsule formulations of *B.bassiana*. Their study revealed that chitosan based capsules exhibited high viability (2.27 x 10^7 cfu mL^{-1}) than talc based ones (1.31 x 10^7 cfu mL^{-}) on 30 DAS.

Gola *et al*. (2019) formulated *B. bassiana* tablets in rice flour and reported 52.5 per cent multimetal removal even after 12 month of storage. Baghel *et al.* (2014) formulated *Trichoderma* tablets using chalk, talc and charcoal as carriers. In their study charcoal was selected a the carrier that supports maximum viability of 7.7 x 10^7 mL^{-1} cfu at 260 days after incubation, whereas the viability in talc based tablet was only 5 x 10^5 mL^{-1} cfu. Chalk based tablets failed to maintain viability. However these attempts were not for the compressed tablets similar to that developed in this study.

Nonetheless, several researchers have standardised suitable carrier materials for EPF formulations, in general. Moslim *et al.* (2009) suggested bran as a carrier for formulating *M. anisopliae* granules and recorded 91.0 ± 5.6 per cent mortality, 20 DAT. Bukhari *et al.* (2011) tested various carrier materials such as wheat flour 0.1% tween 80, white pepper, water savr (sodium bicarbonate), ondina oil 917 and a synthetic oil, shellsol T for formulating *M. anisopliae* and *B. bassiana* and found that shellsol T was the superior carrier as it exhibited 100 per cent mortality of *An. gambiae* larvae in both *M. anisopliae* and *B. bassiana* at 24 HAT.

Another important component of compressed tablets is a binding agent. Among the various binding agents tested in this investigation, carboxy methyl

cellulose (CMC) 7% was superior to microcrystalline cellulose (MCC) 7%, polyvinyl pyrrolidone (PVP) 5% and acacia gum arabic (AG) 5% as it maintained 62.66 per cent germination and 95 per cent mortality on 5 WAS. The corresponding viability and virulence recorded in MCC was 53.82 and 90 per cent, whereas in PVP, it was 39.57 and 70 per cent and in AG it was 43.58 and 77.5 per cent, respectively.

Interestingly, in this study it was noted that the binding agent CMC enhanced the virulence of the formulation (95 % mortality) as a lower death rate was noted in tablets prepared without binding agent (85% mortality). It may be speculated that the increased mortality might be due to better adherence of the conidia to the insect cuticle, imparted by CMC. Raypuriya *et al.* (2019), while studying the compatibility of *M. anisopliae* with different adjuvants, observed that the radial growth of *M. anisopliae* was maximum when the medium was incorporated with CMC 0.5 % and the growth inhibition was minimum, indicating CMC is an excellent binding agent in microbial formulations.

In this study, it was also noted that 5 % PVP reduced the virulence as it recorded only 70 per cent mortality against *Culex* larvae, while in the tablets without binding agent there was 85 per cent mortality. On the contrary, de Medeiros *et al.* (2005) formulated *L. sphaericus* 2362 tablets with 3 % PVP as binder which exhibited good binding properties with 100 per cent virulence against 4th instar *Cx. quinquefasciatus* larvae.

Likewise, addition of MCC 7%, recorded only 53.82 per cent germination 30 DAS, in spite of which it was equally effective as control in terms of viability and virulence against *Culex* larvae. In contrast, Sathyasree *et al.* (2008) reported that 10% MCC used as binding agent in *B. bassiana* tablets using dicalcium phosphate as carrier, there was 90 per cent viability, for more than three months.

One of the major constraints of microbial formulations is the contamination by saprophytic fungi during storage.

The present formulation which included the natural biopolymer, chitosan as the carrier along with talc in the ratio 10 : 90 ratio, was observed to have less contaminants. The number of cfu was limited to 1.2 x 10^5 spores mL^{-1} which was

within the limits prescribed by CIBRC to maintain the quality standards of microbial pesticides. Chitin and its derivative chitosan, not only act as an anti saprophytic agent, but also enhance conidial production as reported by Gerding gonalez *et al*. (2007). *B. bassiana* alginate pellets with chitin 2 % and wheat bran was observed to increase conidiation, whereas higher levels of chitin decreased the number of conidia in pellet. In a study conducted by Abdel - Khader *et al.* (2012), dry formulations of *Trichoderma harzianum* Rifai using sawdust + chitosan was reported to maintain high viability for a period of five months.

The disintegration time of the tablet developed in this study was 8.20 min, and friability noted was 1.5 %. Disintegration time of less than 10 min and friability less than 2% denotes water dispersible characters of a tablet as per the specifications mentioned in Brazilian pharmacopoeia, 4^{th} edition (1988).

5.3 SHELF LIFE OF MICROBIAL FORMULATIONS

One of the major challenges for the success of a microbial formulation is to develop a formulation that can sustain its viability on storage. Moisture content and storage temperature are the critical factors that determines the viability of conidia as well as the extent of contamination during storage. Considering these factors, the compressed tablets developed in this investigation was further subjected to shelf life studies for a period of three months.

The tablets of *M. anisopliae* @ 10^{10} spores mL^{-1}, formulated using talc + chitosan + CMC, at varying moisture levels of 8, 10 and 15 % when subjected to shelf life studies under ambient conditions of storage, revealed that tablets formulated at 15 % were superior in viability (65.06 per cent) and virulence (88 per cent mortality at 8 WAS). Thereafter, though there was a decrease in germination rate below 60 per cent, the virulence could be maintained up to 72 per cent till three months of storage. Tablets with 10 and 8 % moisture recorded 56.93 and 44.46 per cent conidial germination and 78 and 68 per cent mortality, respectively.

Gola *et al.* (2019) studied the shelf life of granules, capsules and tablets of *B. bassiana* in terms of viability and multimetal removal from synthetic waste water over a period of one year. The study indicated that after one year of storage at $30^{o}C$,

granules (2.79 x 10^{10} cfu) and capsules (2.8 x 10^{10} cfu) were more viable than tablets (2.01 x 10^{10} cfu). However highest multimetal removal was exhibited by granules (55.4 per cent), followed by capsules and tablets (39.9 and 35 per cent).

Viability of *M. anisopliae* and *B.bassiana* in chitosan based capsules at varying moisture levels was studied by Remya (2018), revealing that 10% is the ideal moisture content as it maintained both viability and stability. Even though 5% moisture retained the stability of capsule, viability observed was comparatively less, while 15% recorded high viability but reduced stability.

Darakshan *et al.* (2008) reported that viability of *L. lecanii* in talc formulations at 5% and 10% moisture were on par and was significantly higher than that at 15%. This observation disagrees with the findings of this study, where better viability was noted in tablets prepared at 15% moisture content, compared to 10 and 5 %. Anyhow, the results were in agreement with the findings of Baghel *et al.* (2014), who observed that 15% is the ideal moisture level for preparing *Trichoderma tablets.*

In this study it was observed that *M.anisopliae* tablets formulated at 15% moisture content exhibited better shelf life of three months, under room temperature with 72 per cent virulence on *Culex* larvae, compared to 10 % and 8 % levels. Invariably, its storage period could be extended under refrigeration as stated by many researches in the studies cited below.

Daoust *et al.* (1983) observed that viability of spores of *M. anisopliae* was 31 per cent at 37^{o}C at two months after storage and the viability was maintained upto 24 months, when stored under a low temperature of 4^{o}C. Blanford *et al.* (2012) observed that viability of *M. anisolpiae* was 98.1 ± 0.55 per cent when stored at 7^{o}C for a period of two years while it was decreased to 30 per cent when stored at room temperature. Remya and Reji (2021) while studying the shelf life of *M.anisopliae* and *B. bassiana* capsules, it was observed that the shelf life was more when stored under refrigeration. The viability of *M. anisopliae* capsule when stored under refrigeration was maintained as 3.1 x 10^{7} cfu mL^{-1} at 15 DAS while under room temperature it was 2.47 x 10^{7} cfu mL^{-1}. At 75 DAS, the number of cfu recorded was 1.97 x 10^{7} cfu mL^{-1} in capsules stored under refrigeration, while it was 1.88 x 10^{7} cfu mL^{-1} when stored

under room temperature, indicating that refrigeration can extend the shelf life of formulations by 60 days.

5.4 BIOEFFICACY OF TABLETS IN MANAGING MOSQUITO LARVAE

M. anisopliae tablets developed as per the above mentioned standards were validated for their bioefficacy in managing mosquito larvae both under laboratory and semi field conditions. Preliminary studies carried out in the 4^{th} instar larvae of *Culex* under laboratory conditions unveiled that 3 and 4 tablets L^{-1} were equally effective causing 82 and 83 per cent mortality within 9 DAT, with a very low rate of 16 to 17 per cent adult emergence. The adult emergence from untreated larvae was 81 per cent.

Perusal of literature did not reveal any research works that have evaluated the bioefficacy of fungal based tablets for mosquito control. However, efficacy of other formulations of *M. anisopliae* had been reported by various researchers, elsewhere.

Seye *et al.* (2013) reported 28 per cent adult emergence in *An. gambiae* when treated with *M. anisopliae* formulated in neem oil @ 10^7 spores mL^{-1}, under laboratory conditions. Lee *et al.* (2015) revealed fast mosquitocidal activity of granular formulation of *M. anisopliae* JEF-003 @ 10^7 spores mL^{-1} under laboratory conditions in Korea, causing 90 per cent mortality of *Ae. albopicti*, five days post treatment. Bitencourt *et al.* (2018) reported an improved efficiency of formulations over the unformulated spore suspension.. In the laboratory assay carried out using mineral oil formulation of *B. bassiana* CG 479 (10^7 spores mL^{-1}) it was observed that the formulation could cause 83 per cent mortality of *Ae. aegypti* larvae on the seventh DAT, while that observed in conidial suspension was only 56 per cent.

Field level studies carried out using 7, 8, 9 and 10 tablets, revealed that 9 and 10 tablets 10 L^{-1} exhibited similar level of mortality (83.97 and 84.1 per cent) compared to 7 and 8 (75.03 and 79.59 per cent). Therefore, 9 tablet 10 L^{-1} was fixed as the effective dosage for treating stagnant water bodies.

Research reports related to field level evaluation of EPF against mosquito larvae is very scarce. Farenhorst *et al*. (2008) carried out field trials in clay pots with *M. anisopliae,* formulated in Shell ondina oil and recorded its efficacy against adults

of *An. gambiae*. Their studies revealed that spraying 30 mL *M. anisopliae* oil (4 x 10^{10} spores mL^{-1}) in the walls of clay pots, which are the resting sites of mosquitoes recorded 95 ± 1.2 per cent mortality. To evaluate the virulence of *M. anisopliae* on adult mosquitoes, field trials were conducted by Lwetoijera *et al.* (2010) in Tanzania. They prepared *M. anisopliae* baits by impregnating fungal spores @ 3.9 x 10^{10} conidia m^{-2} on black cotton cloths which recorded 95 per cent mortality of *An. arabiens* is adults within 14 days.

Kamalakannan and Murugan (2011) carried out field trials in Tamil Nadu, India for assessing the efficacy of spore suspension of *M. anisopliae* against *Ae. aegypti* larvae. Field application was carried out in 0.5 m^3 tanks @ 2 x 10^8 spores mL^{-1} with the help of a knapsack sprayer which resulted in 100 per cent mortality of larvae on the fifth DAT. In Kenya, Bukhari *et al.* (2011) conducted field trials to study the effect of formulated and unformulated *M. anisopliae* and *B. bassiana* in the pupation of *An. gambiae* larvae. They revealed that *M. anisopliae* and *B. bassiana* formulated in shellsol T exhibited 43 and 39 per cent while that with unformulated conidia was very high recording 84.7 and 72.71 per cent pupation, respectively, indicating a fall in virulence upon formulation.

From the foregoing results, it is obvious that water dispersible tablets of *M. anisopliae* tablets (10^{10} spores mL^{-1}) developed in this study can be effectively used for the management of mosquito larvae in stagnant water bodies as it can self-disperse and release the conidia, with no risk of dustiness. The protocol standardized for tablets can be replicated for other fungi and tested under open field conditions to validate its efficacy. Further, the tablets can be assessed for their shelf life for an extended period of one year.

Summary

6. SUMMARY

The research work entitled “Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control” was conducted at Department of Agricultural Entomology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2019-2021. It included pathogenicity test of entomopathogenic fungi to the common mosquito species found in Kerala, development of water dispersible tablets for the management of mosquito larvae and assessment of their efficacy in managing larvae.

Pathogenicity studies were carried out using the spore suspensions of entomopathogenic fungi (EPF) *Metarhizium anisopliae* (Metsch.) Sorokin NBAIR isolate Ma4, *Beauveria bassiana* (Bals.) Vuillemin NBAIR isolate Bb5, *B. bassiana* KAU isolate ITCC 6063 of 10^8 spores mL^{-1} and *Lecanicillium lecanii* (Zimmerman) Zare and Gams NBAIR isolate Vl 8 and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno KAU isolate ITCC – 7714 of 10^7 spores mL^{-1} in *Anopheles, Aedes* and *Culex.* Symptoms of mycosis, mortality, effective dose, Lethal Concentrations (LC_{50} and LC_{90}) and Lethal Time (LT_{50} and LT_{90}) were worked out.

All the fungi tested were infective to both larvae and adults of mosquitoes at varying levels. Symptoms of mycosis did not exhibit much variation among the species of fungi or mosquitoes tested. Infected larvae were inactive with sinking movement and colour change from dark grey to white. Death occurred within 24 h, with degeneration of gut. Copious amount of mucus around the body was noted after three days of mortality except in *L. saksenae.* Infected adults were inactive and died within 24 h. The cadavers were mummified and found attached to the walls of the container. In Ma4 treated adults, white mycelial growth which turned green upon sporulation was noted 72 h after death.

Observations on mortality taken at 24 h interval revealed that, *M. anisopliae* was superior to all other fungi and exhibited highest mortality to both larvae and adults of all the three mosquito species, followed by *B. bassiana* isolates. *Lecanicillium* spp. were less infective. Larvae of *Anopheles* and *Aedes* when treated with Ma4 exhibited 93.33 and 96.66 per cent mortality, five days after treatment (DAT), which was superior to the biocontrol check, *Bti* (73.33 and 66.66 per cent) and

equally effective as the chemical check malathion 50 EC (100 per cent). Adults were less infective than larvae and recorded 83.33 and 86.66 per cent mortality, which was on par with that observed with *Bti* (66.66 and 76.66 per cent), but inferior to 0.1% malathion 50 EC that recorded 100 per cent mortality. In the case of *Anopheles* the next superior treatment was *B. bassiana* (Bb5) that recorded 83.33 per cent mortality in larve and 56.66 per cent mortality in adults. In *Aedes* both Bb5 and Bb 6063 had similar effect, causing 70 and 76.66 per cent mortality in larvae, whereas in adult only Bb5 was effective (70 per cent mortality). Ma4 was equally effective as malathion 50 EC in *Culex,* causing 100 per cent death in larvae and 96.66 per cent death in adults, five DAT. Unlike in the case of *Anopheles* and *Aedes*, Vl 8 and Bb5 were also effective causing 73.33 per cent and 66.66 per cent death in larvae. Adult mortality recorded with Bb5 was 76.66 per cent, while in Vl 8, it was very low (36.66 per cent).

Dose-mortality studies of *M. anisopliae* carried out using varying concentration of spore suspensions (10^6 to 10^{10} spores mL^{-1}), in the fourth instar larvae, revealed that10^8 spores mL^{-1} was the effective dose causing 95 to 100 per cent mortality in all the three species on the fourth DAT. LC_{90} values computed on the third DAT for *Anopheles, Aedes* and *Culex* was 10^6 spores mL^{-1}. LT_{90} values revealed that *Culex* was more susceptible to *M. anisopliae*, followed by *Aedes* and *Anopheles* the values being 55.9, 79.46 and 90.33 h, respectively. Larva was found to be the most susceptible stage for *Culex,* while for *Anopheles* and *Aedes*, both larva and adult were equally susceptible. Pupa was the least susceptible stage irrespective of species.

Experiment to standardize a suitable carrier material to formulate *M. anisopliae* tablets, revealed that talc + chitosan in the ratio 90:10 was the superior material as there was 71.16 per cent conidial germination and 95 per cent larval mortality in *Culex* on the fifth week after storage (WAS)which was superior to the carriers talc, bran or bran + chitosan (90:10).The corresponding viability and virulence in talc was 66.33 and 87.5 per cent, while in bran it was 43.16 and 70 per cent and in bran + chitosan, it was 47.58 and 80 per cent, respectively.

Among the various binding agents tested, carboxy methyl cellulose (CMC) 7% was superior to microcrystalline cellulose (MCC) 7%, polyvinyl pyrrolidone (PVP) 5% and acacia gum arabic (AG) 5% as it maintained 62.66 per cent

germination and 95 per cent mortality on 5 WAS.The corresponding viability and virulence recorded in MCC was 53.82 and 90 per cent, whereas in PVP, it was 39.57 and 70 per cent and in AG it was 43.58 and 77.5 per cent, respectively.

The tablets of *M. anisopliae* @ 10^{10} spores mL^{-1}, formulated using talc + chitosan + CMC, at varying moisture levels of 8, 10 and 15 % when subjected to shelf life studies under ambient conditions of storage, revealed that tablets formulated at 15 % were superior in viability (65.06 per cent) and virulence (88 per cent mortality at 8 WAS). Thereafter, though there was a decrease in germination rate below 60 per cent, the virulence could be maintained up to 72 per cent till three months of storage. Tablets with 10 and 8 % moisture recorded 56.93 and 44.46 per cent conidial germination and 78 and 68 per cent mortality, respectively. The extent of contamination noticed was significantly high (1.2×10^5cfu mL^{-1}) in 15 % moisture compared to 8 and 10 % moisture levels (4×10^4 and 6×10^4 mL^{-1}). However, 10^5 being the permitted level of contaminants as per CIBRC standards, 15% was fixed as the ideal moisture content for formulating tablets. The effective shelf life of *M. anisopliae* tablets was therefore determined as three months, under ambient conditions of storage.

M. anisopliae tablets were assessed for their bioefficacy to *Culex* sp. under laboratory conditions by observing the larval mortality and adult emergence. Larvae released in tap water treated with water dispersible tablets @ 1, 2, 3 and 4 tablets L^{-1} exhibited mortality from first day onwards. A dosage of 3 and 4 tablets L^{-1} were equally effective causing 82 and 83 per cent mortality within 9 DAT. As the dosage increased from 1 to 4, the rate of adult emergence was found to decrease. Maximum inhibition on emergence was noted in higher doses, where it was 16 to 17 per cent, while 81 per cent of the untreated larvae could pupate and emerge normally. Under field conditions, larvae treated with 7, 8, 9 and 10 tablets 10 L^{-1} exhibited mortality that varied with dosage. Higher dosages of 9 and 10 proved their superiority and were on par with each other, causing 83.97 and 84.1 per cent death at 10 DAT. The corresponding death rate with 7 and 8 tablets 10 L^{-1} was 75.03 and 79.59 per cent. Therefore dosage of *M. anisopliae* tablets for mosquito management in stagnant water was determined as 9 tablet 10 L^{-1}.

It is concluded that water dispersible tablets of *M. anisopliae* formulated at 10^{10} spores mL^{-1} with talc + chitosan + CMC (7%) at 15% moisture is effective for the management of mosquito larvae in stagnant water bodies @ 9 tablets 10 L^{-1}. They can be stored effectively for three months with 72 per cent virulence, under ambient conditions.

The salient findings of the investigation are

- Entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* are infective to the larvae and adults of common mosquito species *Anopheles, Aedes* and *Culex,* while *Lecanicillium* spp was less infective.
- Effective dose of *M. aniopliae*, the most effective fungus was 10^8 spores mL^{-1}
- *Culex* was more susceptible to *M. anisopliae* compared to *Anopheles* and *Aedes.*
- LC_{50} value of *M. anisopliae* was 3.75 x 10^2, 3.3 x 10^3 and 3.75 x 10^2 spores mL^{-1} to *Anopheles, Aedes* and *Culex*.
- LT_{90} was lowest for *Culex* (55.9 h) compared to *Aedes* (79.46 h) and *Anopheles* (90.33 h).
- *Culex* is more susceptible species to fungal infection and larva is the most susceptible stage.
- Talc + chitosan (90:10) is the best carrier material that could retain viability and virulence.
- Carboxy methyl cellulose 7% is the superior binding agent for water tablet formulation.
- A moisture content of 15 % is the ideal for maintaining the viability and virulence of tablets.
- Water dispersible tablets of *M. anisopliae* can be stored under ambient conditions for a period of three months.
- Dosage of tablets for managing mosquito larvae in stagnant water is 9 tablets 10 L^{-1}.

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TABLET FORMULATION OF ENTOMOPATHOGENIC FUNGUS AND ITS BIOEFFICACY IN MOSQUITO CONTROL

by

NEEMA DILEEP

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Abstract of the thesis

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ABSTRACT

The research work entitled “Tablet formulation of entomopathogenic fungus and its bioefficacy in mosquito control” was conducted at Biocontrol Laboratory for Crop Pest Management, Department of Agricultural Entomology, College of Agriculture, Vellayani, Thiruvananthapuram, during the year 2019-2021 with an objective to develop water dispersible tablets of entomopathogenic fungus and to test their effectiveness in managing mosquitoes.

Pathogenicity studies carried out using 10^8 spores mL^{-1} of *Metarhizium anisopliae* (Metsch.) Sorokin NBAIR isolate Ma4, *Beauveria bassiana* (Bals.) Vuillemin NBAIR isolate Bb5, *B. bassiana* KAU isolate ITCC 6063 and 10^7 spores mL^{-1} of *Lecanicillium lecanii* (Zimmerman) Zare and Gams NBAIR isolate Vl 8 and *Lecanicillium saksenae* (Kushwaha) Kurihara and Sukarno KAU isolate ITCC – 7714, revealed that all the fungi tested were infective to *Anopheles*, *Aedes* and *Culex* at varying levels. Infected larvae were less active at 12 hour after treatment (HAT) with sinking movement and colour change from dark grey to white. Death occurred within 24 h, with degeneration of gut. Copious amount of mucus was noticed around the body, except in *L. saksenae.* Infected adults were inactive and died within 24 h. The cadavers were mummified and found attached to the walls of the container. In Ma4 treated adults, white mycelial growth which turned green upon sporulation was noted 72 h after death.

Observations on mortality taken at 24 h interval revealed that, *M. anisopliae* was the most effective fungus for mosquito control, followed by *B. bassiana* isolates. *Lecanicillium* spp. were less effective to mosquitoes. *M. aniospliae* was found to be superior to the biocontrol check, *Bti* and equivalent to the chemical check malathion 50 EC based on larval mortality. Mortality recorded by *M. anisopliae* was 93.33, 96.66 and 100 per cent in *Anopheles*, *Aedes* and *Culex* respectively, 5 days after treatment (DAT), while in *Bti* it was 73.33, 66.66 and 70 per cent respectively. The corresponding mortality in malathion 50 EC was 100 per cent. Based on mortality recorded in adults, *M. anisopliae* (83.33 and 86.66 per cent mortality) was equally effective as *Bti* to *Anopheles* and *Aedes* (66.66 and 76.66 per cent mortality) but inferior to malathion 50 EC that recorded complete mortality. The mortality recorded

in *Culex* was 96.66 per cent, which was on par with that recorded in malathion 50 EC (100 per cent).

Dose-mortality studies of *M. anisopliae* on 4th instar larvae, revealed that 10^8 spores mL^{-1} was the effective dose. The LC_{90} values for *M. anisopliae* was 10^6 in *Anopheles*, *Aedes* and *Culex*. The LT_{90} values were 90.33, 79.46 and 55.9 h on *Anopheles*, *Aedes* and *Culex*, revealing that *Culex* is the most susceptible species. Larva was found to be the most susceptible stage for *Culex*, while for *Anopheles* and *Aedes*, both larva and adult were equally susceptible, whereas pupa was the least susceptible stage for all the three species.

Experiment to standardize the carrier material for *M. anisopliae* tablets, revealed that talc + chitosan (90:10) was superior to bran, talc and bran + chitosan, as there was 71.16 per cent conidial germination and 95 per cent larval mortality in *Culex* on the 5th week after storage (WAS). The corresponding viability and virulence in talc were 66.33 per cent and 87.5 per cent respectively, while in bran it was 43.16 and 70 per cent and in bran + chitosan, it was 47.58 and 80 per cent, respectively. Among the binding agents tested, Carboxy Methyl Cellulose (CMC) 7% was superior to Microcrystalline Cellulose (MCC) 7%, Polyvinyl Pyrrolidone (PVP) 5% and Acacia Gum Arabic (AG) 5% as it maintained 62.66 per cent germination and 95 per cent mortality on 5 WAS.

The tablets of *M. anisopliae* @ 10^{10} spores mL^{-1}, formulated using talc + chitosan + CMC 7% at varying moisture levels of 8, 10 and 15 % when subjected to shelf life studies revealed that tablets formulated at 15 % were superior in viability (65.06 per cent) and virulence (88 per cent mortality) at 8 WAS. Thereafter, though there was a decrease in germination rate below 60 per cent, the virulence could be maintained up to 72 per cent till three months of storage. Extent of contamination noticed was significantly high (10^5 cfu mL^{-1}) in 15 % moisture compared to 10^4 in 8 and 10 % moisture levels. However, 10^5 being the permitted level of contaminants as per CIBRC standards, 15% was fixed as the ideal moisture content for formulating tablets. The effective shelf life was therefore determined as three months under ambient conditions.

The tablets when tested for their bioefficacy to *Culex* larvae revealed that 3 and 4 tablets L^{-1} were equally effective causing 82 and 83 per cent mortality within 9 DAT, under laboratory conditions. The adult emergence from the treated larvae was 17 and 16 per cent for 3 and 4 tablets L^{-1}, which was significantly lower than that from control (81 per cent). Under field conditions, 9 and 10 tablets 10 L^{-1} exhibited similar level of mortality (83.97 and 84.1 per cent) compared to 7 and 8 (75.03 and 79.59 per cent). Therefore 9 tablet 10 L^{-1} was fixed as the effective dosage for treating stagnant water bodies.

It is concluded that water dispersible tablets of *M. anisopliae* formulated at 10^{10} spores mL^{-1} with talc + chitosan + CMC (7%) at 15% moisture is effective for the management of mosquito larvae in stagnant water bodies @ 9 tablets 10 L^{-1}. They can be stored effectively for three months with 72 per cent virulence, under ambient conditions.

സംഗ്രഹം

വെള്ളായണി കാർഷിക കോളേജിലെ എന്റോമോളജി വിഭാഗത്തിൽ 2019–2021 കാലയളവിൽ നടത്തിയ "കൊതുകുകളെ നിയന്ത്രിക്കാൻ മിത്രകുമിളുകൾ ഉപയോഗിച്ചുള്ള ടാബ്‌ലറ്റുകൾ" എന്ന ഗവേഷണ പദ്ധതിയുടെ പ്രധാന ഉദ്ദേശങ്ങളും കണ്ടെത്തലുകളുമാണ് ചുവടെ പ്രതിപാദിച്ചിരിക്കുന്നത്.

മിത്രകുമിളുകളായ മെറ്റാറൈസിയം അനൈസോപ്ലിയെ, ബിവേറിയ ബാസിയാന, ലെക്കാനിസിലിയം ലെക്കാനി, ലെക്കാനിസിലിയം സക്സേനെ എന്നിവ കേരളത്തിൽ സാധാരണയായി കണ്ടുവരുന്ന കൊതുക് ഇനങ്ങളായ അനോഫെലിസ്, ഈഡിസ്, കൂലക്സ് എന്നിവയ്ക്ക് ഫലപ്രദമാണോയെന്ന കണ്ടെത്തുക എന്നതായിരുന്ന പ്രധാന ലക്ഷ്യം. ഈ മിത്രകുമിളുകളുടെ പ്രവർത്തനശേഷി വിലയിരുത്തുന്നതിന് ഡോസ്, LC_{50}, LC_{90}, LT_{50}, LT_{90} എന്നിവ കണ്ടെത്തിയിരുന്നു. ഇവയിൽ ഏറ്റവും ഉത്തമമായ മിത്രകുമിൾ ഉപയോഗിച്ച് ടാബ്‌ലറ്റുകൾ എങ്ങനെ തയാറാക്കാം എന്നതായിരുന്ന ഈ പഠനത്തിന്റെ ഒരു പ്രധാന ഭാഗം. ഇപ്രകാരം വികസിപ്പിച്ചെടുത്ത ടാബ്‌ലറ്റുകൾ കെട്ടിക്കിടക്കുന്ന വെള്ളത്തിൽ നിക്ഷേപിച്ച് അവയ്ക്ക് കൊതുകുകളെ നശിപ്പിക്കുന്നതിലുള്ള കാര്യക്ഷമത വിലയിരുത്തുക എന്നതായിരുന്ന ഈ പദ്ധതിയുടെ മറ്റൊരു ഉദ്ദേശം.

പരീക്ഷണശാലയിൽ നടത്തിയ പ്രാഥമിക പഠനങ്ങളിൽ നിന്നും മെറ്റാറൈസിയം അനൈസോപ്ലിയെ, ബിവേറിയ ബാസിയാന എന്നിവയ്ക്ക് കൂത്താടികളേയും പൂർണ വളർച്ചയെത്തിയ കൊതുകുകളേയും നശിപ്പിക്കാൻ കഴിയുമെന്ന് ബോധ്യപ്പെട്ടു. ഈ മിത്രകുമിളുകൾക്ക് 66.66 മുതൽ 100% വരെ കൂത്താടികളെ 5 ദിവസത്തിനകം നശിപ്പിക്കാനുള്ള കഴിവുള്ളതായും 56.66 മുതൽ 96.66% വരെ പൂർണ്ണവളർച്ചയെത്തിയ കൊതുകുകളെ നശിപ്പിക്കാൻ കഴിവുള്ളതായും കണ്ടെത്തി. രോഗം പിടിപെട്ട കൂത്താടികളുടെ അന്നനാളം ദ്രവിച്ച് പോകുന്നതായും ചുറ്റും ഒരു കൊഴുത്ത ദ്രാവകം ആവരണം ചെയ്യപ്പെട്ടതായും കണ്ടിരുന്നു. രോഗം ബാധിച്ച കൊതുകുകളിൽ 3 ദിവസത്തിൽ കുമിൾ വിത്തുകൾ മുളച്ച് വരുന്നതും കണ്ടിരുന്നു.

തുടർന്നുള്ള പരീക്ഷണങ്ങൾക്കായി ഒരു ടാബ്‌ലറ്റ് പ്രസ് സ്വന്തമായി രൂപപ്പെടുത്തുകയും ഉണ്ടാക്കുകയും ചെയ്തിരുന്നു. ഇതുപയോഗിച്ച് പ്രവർത്തനശേഷിയിൽ മികച്ചതായി കണ്ടെത്തിയ മെറ്റാറൈസിയം അനൈസോപ്ലിയെ എന്ന മിത്രകുമിളിന്റെ ഉയർന്ന കോൺസെൻട്രേഷനിലാണ് (10^{10} spores mL^{-1}) ടാബ്‌ലറ്റുകൾ നിർമിച്ചത്. ഇതിലേക്ക് ആവശ്യമായ കാരിയർ കണ്ടെത്തുക എന്ന പരീക്ഷണത്തിൽ ടാൽക്കും കൈറ്റോസാനും 90:10 എന്ന അനുപാതത്തിൽ ചേർത്ത് തയാറാക്കുന്നത് നല്ലതാണെന്നു കണ്ടെത്തി. ഇതോടൊപ്പം ചേർക്കേണ്ട ബൈൻഡിങ് ഏജന്റുകൾ പരീക്ഷിച്ചതിൽ 7% കാർബോക്സി മീതൈൽ സെല്ലുലോസ് ആണ് ഉത്തമമെന്നും കുമിൾ വിത്തുകളുടെ അംഗ്രണശേഷിയിൽ നിന്നും മനസ്സിലാക്കി. 15% ജലാംശം അടങ്ങിയ ടാബ്‌ലറ്റുകൾക്ക് കുമിളുകളുടെ അംഗ്രണശേഷി നിലനിർത്താൻ കഴിയുമെന്നും

സാധാരണ ഊഷ്മാവിൽ 3 മാസം വരെ സൂക്ഷിച്ച് വയ്ക്കാൻ അനുയോജ്യമാണെന്നും കണ്ടെത്തി.

മേൽ വിവരിച്ച പ്രകാരം തിട്ടപ്പെടുത്തിയ ഘടകങ്ങൾ ഉപയോഗിച്ച് തയാറാക്കിയ "കംപ്രസ്ഡ് ടാബ്ലറ്റുകൾ" കൊതുകുകളുടെ കൂത്താടിക്കെതിരെ ഉറസ്സായ സ്ഥലത്ത് വെള്ളം കെട്ടിനിർത്തി പരീക്ഷിക്കുകയുണ്ടായി. ഈ പരീക്ഷണത്തിൽ നിന്നും 10 ലിറ്റർ കെട്ടിക്കിടക്കുന്ന വെള്ളത്തിൽ 9 ടാബ്ലറ്റ് (9 ഗ്രാം) നിക്ഷേപിച്ചാൽ 10 ദിവസ്സം കൊണ്ട് 84% കൂത്താടികളെ നശിപ്പിക്കാൻ കഴിയുമെന്ന് കണ്ടെത്തി.