SELECTION FOR ABIOTIC STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* SOROKIN

by

JANCY MERLIN JOHNSON

(2017-11-008)

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

2020

SELECTION FOR ABIOTIC STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* SOROKIN

by

JANCY MERLIN JOHNSON

(2017-11-008)

THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(AGRICULTURAL ENTOMOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2020

DECLARATION

I hereby declare that this thesis entitled **"Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin"** is a bona fide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title of any other university or society.

Vellanikkara
Date: 09-06-2020

Jancy Merlin Johnson
(2017-11-008)

CERTIFICATE

Certified that this thesis entitled **"Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin"** is a bona fide record of research work done independently by **Ms. Jancy Merlin Johnson (2017-11-008)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellanikkara
Date: 09-06-2020

Dr. Deepthy K. B.
(Chairman, Advisory Committee)
Assistant Professor
Department of Agricultural Entomology
College of Hoticulture
Vellanikkara, Thrissur

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Jancy Merlin Johnson (2017-11-008)**, a candidate for the degree of **Master of Science in Agriculture** with major field in **Agricultural Entomology**, agree that the thesis entitled **"Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin"** may be submitted by **Ms. Jancy Merlin Johnson** in partial fulfillment of the requirement for the degree.

Dr. Deepthy K. B.
(Chairman, Advisory Committee)
Assistant Professor
Department of Agricultural Entomology
College of Horticulture
Vellanikkara

Dr. Mani Chellappan
(Member, Advisory Committee)
Professor and Head
Department of Agricultural Entomology
College of Horticulture
Vellanikkara

Dr. Madhu Subramanian
(Member, Advisory Committee)
Professor and Head
AICRP on BCCP
Vellanikkara

Dr. Reshmy Vijayaraghavan
(Member, Advisory Committee)
Assistant Professor
Department of Plant Pathology
College of Horticulture
Vellanikkara

ACKNOWLEDGEMENT

First and foremost, praises and thanks to ***Almighty*** *for giving me strength, knowledge, ability and opportunity to undertake this research work and to persevere and complete it satisfactorily. I would like to take this opportunity to thank everyone who have supported me throughout my research programme.*

It is with immense pleasure, i would like to express my deep sense of whole hearted gratitude, indebtedness and heartfelt thanks to my major advisor ***Dr. Deepthy K, B.****, Assistant Professor, Department of Agricultural Entomology for her unreserved help, guidance, affectionate advices, abiding patience and above all, I gratefully remember instilling love of learning, wholehearted co-operation and motherly approach she gave me throughout the course of my study.*

I owe my most sincere gratitude to ***Dr. Mani Chellappan****, Professor and Head, Department of Agricultural Entomology and member of my advisory committee for the timely help, friendly demeanour, valuable advice and constant support rendered during the course of study.*

I am deeply obliged to ***Dr. Madhu Subramanian****, Professor and Head, AICRP on BCCP, Vellanikkara and member of my advisory committee for his valuable suggestions and help for thesis writing, constructive criticisms and also for providing me the facilities at BCCP laboratory during my research work.*

I am extremely indebted to ***Dr. Reshmy Vijayaraghavan****, Assistant Professor, Department of Plant Pathology and member of my advisory committee for her well-timed guidance and help extended during the research work and also for allowing me to use the facilities at Pathology laboratory during my research work.*

I am also grateful to ***Dr. Haseena Bhasker****, Professor (AINPAA), Department of Agrl. Entomology and* ***Dr. Berin Pathrose****, Assistant Professor, Department of Agrl. Entomology for*

their excellent support, encouragement and also providing me the facilities at Acarology and pesticide residue laboratories during my research work.

My heartfelt thanks to ***Dr. Smitha Ravi****, Assistant Professor, Department of Agricultural Entomology for her friendly approach, valuable assistance, generosity in clearing doubts offered during my research period.*

I am very much obliged and grateful to my teachers ***Dr. Smitha M. S., Dr. Vidya C. V.*** *and* ***Dr. Ranjith M. T.*** *for their kind treatment, moral support and encouragement offered throughout the work.*

I am extremely grateful to ***Dr. Anitta Cherian,*** *Professor and Head, Dept. of Plant Pathology,* ***Dr. Jayasree****, Professor and Head, Dept. of Soil science and Agrl. Biochemistry,* ***Dr. K. P. Prameela****, Professor, Dept. of Agronomy,* ***Dr. Girija****, Professor and Head, Dept. of Plant Physiology,* ***Dr. Beena****, Assistant Professor, Dept. of Soil Science and Agrl. Biochemistry,* ***Dr. Beena****, Assistant Professor, AICRP on Medicinal Plants,* ***Dr. Laly C. John,*** *Professor and Head, Department of Agricultural Statistics for their valuable help during my research work.*

I would like to thank in depth to ***Dr. C. George Thomas****, Former Associate Dean, COH, Vellanikkara for his valuable suggestions in thesis writing. I owe special thanks to* ***Dr. C. Narayanankutty****, Associate Dean, COH, Vellanikkara,* ***Dr. A. T. Francis****, Librarian, COH and all other staff members of the Central Library. I also thankfully remember the services rendered by staff members of the Student's computer club, Library and Office of College of Horticulture, Vellanikkara. I am thankful to the* ***Kerala Agricultural University*** *for technical and financial assistance for persuasion of my study and research programme.*

I express my sincere thanks to the ***Department of Agricultural Entomology*** *and the non-teaching staffs, especially* ***Rama chechi, Surya chechi*** *and* ***Presanna chichi*** *for their motherly affection, cooperation and support extended throughout the study period.*

I am also thankful to Sreeja miss (Dept. of Entomology), Malithi miss (Dept. of Soil Science and Agrl. Biochemistry), Milsha chechi (Dept. of Plant Pathology), Henna chechi (Ornithology), Nabeesa itha, Reshma chechi, Akhila chechi, Shaly chechi, Jisna chechi, Gokul

chettan, Sarath chettan (AICRP on BCCP), Ancy chechi,(AINPAA), Akhil and Anisha (Pesticide Residue Analysis Laboratory), Nishad chettan, (College of Forestry) for their timely help and cooperation given during the research period.

It's my fortune to gratefully acknowledge the infinite affection, warm concern, constant encouragement and moral support of classmates Anna and Anusree and also to my juniors Sachin, Pavithra Kumar, Salma, Sravanthi, Laya, Karthika and Abinsha. I am genuinely indebted to my seniors Stella chechi, Nimisha chechi, Anusree chechi, Shahanaz chechi, Vineetha chechi, Kavya chechi, Athira chichi, Vandhana chechi, Anjaly chechi and Sharanbasappa chettan for their words of support and guidance during the entire period of my research.

I would like to extend my huge, warm thanks to my dear friends Reshma, Sarga, Anjaly, Chinju, Athulya, Mekha, Sabika, Linu, Anu, Jayalakshmi, Anusha, Agina, Alfiya, Emily, Athira, Induja, Jazeel, Anirudh, Haritha Raj, Anasooya, Nayana, Sindhuja, Sreedevi, Aliya, Anush and Jobin for their generous help and mental support rendered to me during the research work.

I am deeply indebted to my father ***Johnson Joseph****, my mother* ***Annamma Johnson*** *and my sister Ancy Johnson for their unconditional love, constant support, prayers, blessings and understanding even at extended span of study without which I would never have completed this venture. Once again, I express my sincere gratitude to all those who contributed in many ways to the success of this study and made it an unforgettable experience.*

Jancy Merlin Johnson

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Pest problems are almost an unavoidable part of crop production. In the pre insecticide era, farmers relied on the natural biocontrol and innate resistance of the crop for the control of pests. With the discovery of insecticides, insect pest management had been dominated by the use of synthetic pesticides. Serious environmental concerns and increased health risks associated with the indiscriminate use of synthetic insecticides have led to intensified efforts to develop suitable biological control strategies for integrated pest management.

Entomopathogenic fungi (EPF) are fungal microorganisms that are pathogenic to insects. Insect pathogenic fungi play a crucial role in regulating insect population as pest control agents. The prospects of using them as an alternative to synthetic chemicals paved an effective and relatively safer way for pest management. The green muscardine fungus, *Metarhizium anisopliae* is one of the most widely used biocontrol agent and has been found to be effective against several species of insects including beetles, termites, lepidopterans, leafhoppers and mosquitoes. Performance of mycoinsecticides in the field condition mainly relied on their potential to overcome abiotic stress conditions. Like other entomopathogenic fungi, *Metarhizium* is also prone to abiotic stresses which markedly reduce their potential as biocontrol agents. It has been recovered from a variety of crop ecosystems rendering it an ideal candidate for exploration on stress tolerant isolates.

Soil abiotic factors like temperature, pH, moisture content, minerals and organic matter content can influence fungal persistence and its activity (Asensio *et al*., 2003). Poor field performance under environmental stress conditions coupled with high cost of production, affected successful marketing and usage of mycoinsecticides. Most of the formulations of *Metarhizium anisopliae* available in markets are only suitable for favourable conditions. Therefore, there is an urgent need to identify potential *Metarhizium anisopliae* isolates which can sustain fluctuating

soil moisture regimes, varying temperatures, saline, acidic conditions and pesticide induced stress. Isolates that are capable to survive such stress conditions will retain their biocontrol potential and will be effective in managing pests in adverse environmental conditions. Hence, this study is proposed to identify different abiotic stress tolerant isolates of *M. anisopliae* from Kerala soils and to study their potential for performance under adverse environmental conditions. The study includes the following objectives:

- ✓ Isolation and identification of *M. anisopliae* from different stressed locations
- ✓ *In vitro* screening of *M. anisopliae* isolates for stress tolerance
- ✓ Biochemical characterization of stress tolerant isolates of *M. anisopliae*
- ✓ *In vitro* evaluation of stress tolerant isolates of *M. anisopliae* for their biocontrol efficacy

Review of literature

2. REVIEW OF LITERATURE

Environmental contamination and increased health risks associated with the use of synthetic chemical pesticides have oriented researchers to concentrate their efforts for developing successful biological control agents for their deployment in integrated pest management (IPM) programme. Entomopathogenic fungi are important candidates in pest management, however their efficacy as biocontrol agent are constrained in adverse abiotic stress conditions. The green muscardine fungus, *Metarhizium anisopliae* has been recovered from a variety of crop ecosystems rendering it an ideal candidate for exploration on stress tolerant isolates. A perusal of literature pertaining to the topic "Selection of abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin" are described in this chapter.

2.1. PHYSICO-CHEMICAL PROPERTIES OF SOIL SAMPLES COLLECTED FROM STRESSED LOCATIONS OF KERALA

Microbial diversity in the soil is greatly influenced by the soil reactions and its chemical properties. Indira and Covilakam (2013) reported that Kuttanad tracts of Kerala showed remarkable acidic pH in the range of 2.5 to 5.2. Similarly, Thrissur soils were also showed a pH ranging from 2.9 to 7.7, designating extremely acidic to slightly alkaline in reaction (Kavitha and Sujatha, 2015).

Beevi *et al*. (2014) conducted a study on pesticide residues of soil under cardamom plantations of Idukki district. Out of 100 samples analysed, 70 per cent of samples were contaminated with DDT, endosulfan, imidacloprid, cypermethrin and ethion. Similar results were also reported by Jacob *et al*. (2015), in which majority of the cardamom plantations of Idukki were contaminated with persistent pesticide residues of DDT, endosulfan and organophosphorus pesticides.

Mohan and Sreelatha (2016) studied the nutrient dynamics of pokkali padashekarams of RRS, Vyttila and revealed that the soil pH was higher during

harvest with a value of 4.26 compared to the mound preparation stage with pH 3.9 and the electrical conductivity decreased from mound preparation to harvest stage.

2.1.1. Isolation of *Metarhizium anisopliae* Sorokin

Raid and Cherry (1992) reported that the conidia of *Metarhizium anisopliae* were pathogenic under a wide range of temperature and soil moisture. Hallsworth and Magan (1999), Faria *et al*. (2009) and Faria *et al*. (2015) suggested that conidial viability had been the main parameter used to determine the effects of various environmental factors on entomopathogenic fungi, including solar radiation, dehydration and water stress, extreme temperatures, pH, vigour and imbibition damage of conidia. *Metarhizium anisopliae* is a typical soil borne fungus and various studies had been made on its isolation, growth and morphological characteristics in different media.

Veen and Ferron (1966) developed a selective medium called Veen's medium for the isolation of *M. anisopliae* from soil, consisting of glucose, peptone, oxgall as nutrient source, selective fungicide dodine (*N*-dodecylguanidine monoacetate), actidione or cycloheximide as antifungal antibiotic and chloramphenicol as bacterial antibiotic. Milner *et al*. (1992) suggested that Veen's media with 10 µg/ml dodine was found to be the best for the isolation of *Metarhizium* spp. from soil. Liu *et al*. (1993) also studied the effect of dodine in Veen's medium at different concentrations on germination and colony production of *Metarhizium* spp. and found that low concentrations (10 to 50 µg/ml) of dodine resulted in the selective isolation of *Metarhizium* from the soil by reducing the contaminants to 10 per cent.

Kmitowa *et al*. (1977) used greater wax moth, *Galleria mellonella* as a test insect in the bait method due to higher susceptibility to most of the fungal pathogens. Zimmermann (1986) and Keller *et al*. (2003) described *Galleria* bait method for the detection of entomopathogenic fungi from soil and revealed the method as a simple feasible tool, offering opportunities for exploring occurrence and distribution of

entomopathogenic fungi and their strains. Other researchers also employed *Galleria* larva as a bait for the isolation of entomopathogenic fungi from soil (Bidochka *et al*., 1998; Klingen *et al*., 2002; Neuman and Shields 2004; Meyling and Eilenberg, 2006; Sun *et al*., 2008; Baverstock *et al*., 2010; Rishi *et al*., 2013).

Fernandes *et al*. (2010) reported a dodine-free selective medium, named as CTC medium, consisting of potato dextrose agar + yeast extract (PDAY) supplemented with chloramphenicol, thiabendazole and cycloheximide and found to be efficient in isolating *Metarhizium* spp., and *Beauveria* spp. from soil. Posadas *et al*. (2012) also reported Oat meal agar medium with chloramphenicol and Cetyl Trimethyl Ammonium Bromide (CTAB) as a common medium for the isolation of *B. bassiana*, *M. anisopliae* and *Paecilomyces* from soil.

Hasan *et al*. (2012) employed serial dilution technique for the isolation of fungi from soil. Sowmya (2017) demonstrated different techniques for the isolation of *M. anisopliae* from soil and insect samples. According to the study, soil serial dilution method and plating of surface sterilized insects were founded to be best isolation technique.

Liu *et al*. (2007) suggested that potato dextrose agar medium was commonly used as a medium to culture *M. anisopliae.* Keppanan *et al*. (2018) used *Galleria* bait method for the isolation of *M. anisopliae* in sampling region of Western Ghats. They also practiced solid-state fermentation to optimize the mass production of *M. anisopliae* and enhanced their virulence against insect pest.

2.1.1.1. Selection of Suitable Cultural Medium for Metarhizium anisopliae

Liu *et al*. (2012) observed colony morphology and conidial yield of *M. anisopliae* on three agar based media like Potato Dextrose Agar medium (PDA), PDA + Peptone (1 %) (PPDA) and Oat Meal Agar Medium (OMA). The results revealed that PPDA was the excellent culture medium for colony growth and conidial yield. The average conidial yield of *M. anisopliae* was 1.02×10^9 conidia/ml on

PPDA which was significantly more than that of PDA medium (3.81×10^8 conidia/ml) and OMA medium (1.96×10^8 conidia/ml).

Patil *et al*. (2014) evaluated growth, development and viability of *M. anisopliae* on different culture media like Sabouraud's Dextrose broth + Yeast Extract (SDYE), Sabouraud's Maltose broth + Yeast Extract (SMYE), Potato Peptone broth (PP), Potato Maltose broth (PM), Potato Glucose broth (PG), Potato Dextrose broth (PD), Potato Dextrose broth + Yeast Extract (PDYE), Yeast Extract Glucose broth (YEG) and Malt Extract broth (ME). The study proved that Sabouraud's dextrose broth with yeast extract promoted better growth of *M. anisopliae*, attaining cent per cent surface coverage at three DAI. It was followed by sabouraud's maltose broth + yeast extract, Potato dextrose broth + yeast extract and potato dextrose broth. Im *et al*. (1988) found that dextrose was necessary for sporulation and yeast for mycelial growth.

2.1.1.2. Characterization of Metarhizium anisopliae Isolates

According to Kamath *et al*. (1952), spore size of *M. anisopliae* was influenced by cultural medium on which it was grown. Ghayedi and Abdollahi (2013) described *M. anisopliae*, having yellowish green, olivaceous, dark-herbage green with pink or vinaceous buff growth.

Bridge *et al*. (1993) conducted a study on the morphological, biochemical and molecular characteristics of *M. anisopliae* and observed that the dark-green colour of spore mass was more characteristic of *M. anisopliae*. Sepulveda *et al*. (2016) also demonstrated the morphological character of *M. anisopliae*, having hyaline olive green coloured cylindrical conidia with round edges.

Samson *et al*. (1988) characterized *M. anisopliae* and reported that it had cylindrical conidiogenous cells called phialides, forming chains of conidia which continually aggregated into prismatic columns. Glare *et al*. (1996) also studied the phialide morphology of 11 isolates of genus *Metarhizium* and observed that phialide

morphology of a single isolate keep varying within the same culture as well as between different substrates.

Tulloch (1976) described two varieties on the basis of conidial length. *Metarhizium anisopliae* var. *anisopliae* had straight sided conidia of 3.5 to 9.0 µm in length whereas *M. anisopliae* var. *majus* had larger conidia measuring 9.0 to 18.0 µm. Similarly, Liu *et al*. (2012) studied and characterized *M. anisopliae* var. *anisopliae* as cylindrical with obtuse end, slightly narrowing at the center with conidial width of 1.5 to 3 µm and length of 4 to 8 µm.

Fernandes *et al*. (2010) noticed the cultural characters of *M. anisopliae* on potato dextrose agar medium with edges white with varying thickness while the reverse side of Petri plate showed brownish, orange and yellow or white growth. However, the morphological and cultural characters of the colonies could differ depending upon the media type (Kamp and Bidochka, 2002), aging (Wang *et al*., 2013) and other factors. Brunner-Mendoza *et al*. (2019) also reported the cultural characters of *M. anisopliae* on potato dextrose agar medium as dark green, light green white or brownish, or even as bicoloured growth in the centre.

2.1.1.2. Purification and Preservation of Metarhizium anisopliae Isolates

Preservation of fungal cultures is an essential aspect for carrying out future studies. Most common methods of preservation of fungal cultures include preservation by serial transfer, storing in sterile water, mineral oil, lyophilization (freeze drying) (Butt *et al*., 2001) and cryopreservation (freeze drying with liquid nitrogen).

Onions (1983) reported recovery of an isolate of *M. anisopliae* after ten years of storage under lyophilization, with good growth and conidia production. Ayala-Zermeno *et al*. (2017) also evaluated the viability, purity and genetic stability of entomopathogenic fungi using different preservation methods. The effective methods

of preservation for EPF were cryopreservation at -196°C, ultra cold freezing at -70°C, lyophilization and silica gel preservation.

Similarly, Toegel *et al*. (2010) assessed the effect of lyophilisation on the conidia and biomass of *Beauveria brongniartii* and *M. anisopliae*. The conidia of *M. anisopliae* showed sensitivity to the drying process while *B. brongniartii* showed high sensitivity to ultra-freezing. So they used fructose, glucose and sucrose as cryoprotectants, which improved the viability of the conidia after lyophilisation. Moreover, the combination of dextran 4 with fructose gave good physical characteristics to the fungal biomass. It also preserved the stability of the production of destruxin A and destruxin B of *M. anisopliae* and oosporein of *B. brongniartii*.

Cavalcanti (1991) and Silva *et al*. (1994) revealed that fungal cultures remained viable for decades when kept under mineral oil. Lastra *et al*. (2002) described the preservation methods using mineral oil and sterile water and observed that these methods were excellent for preserving fungi of the order Hypocreales. After 18 months of preservation, they recovered viable isolates of *M. anisopliae, Paecilomyces fumosoroseus, Hirsutella thompsonii* and *Verticillium lecanii*.

Experiments done by Bell and Hamalle (1974), Smith (1993) and Windels *et al*. (1993) revealed that silica gel based preservation technique of fungal spores proved as a valid and inexpensive method for maintaining spore viability of entomopathogens for more than ten years.

According to Humber (1997), preservation of entomopathogens coming under entomophthorales in sterile distilled water was convenient. Kitamoto *et al*. (2002) reported that preservation of fungal culture for longer period was a complicated process and it could be done by continuous subculture method and storing at 4°C. Paul *et al*. (2015) detailed long term preservation of fungi in glycerol at 4°C. According to him, preserving fungi in glycerol (15 %) at 4°C in vials and glycerol (50 %) in PDA slants had exhibited viability upto 24 months.

2.2. *IN VITRO* SCREENING FOR ABIOTIC STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae*

Various abiotic stresses like high temperature, acidity, salinity, drought *etc* might induce changes in structure and metabolism of an organism and results in synthesis of stress specific compounds which could help microorganisms in surviving the situation.

2.2.1. Acidity Tolerance

Teja and Rahman (2017) assessed the effect of pH levels of 4 to 9 on the growth of different entomopathogenic fungi and observed that *M. anisopliae* isolates showed maximum fungal growth at pH level of 4 to 5. It was also found that pH had a great influence on the mass production of EPF in large scale, hence makes it necessary to evaluate tolerance of different entomopathogens to pH.

Hallsworth and Magan (1996) noticed growth of some entomopathogenic fungi like *B. bassiana, M. anisopliae* and *P. farinosus* at different pH levels and concluded that EPF species could grow over a broad range of pH from 2.5 to 10.5 and the growth was optimum at pH range of 5 to 8.

Kotwal *et al*. (2012) reported the influence of different physical parameters on growth and sporulation of *M. anisopliae*. In case of pH at different levels of 5.5, 6, 6.5, 7, 7.5, 8 and 8.5, highest radial growth of mycelia was obtained at pH 5.5 with colony diameter of 35.66 mm in Sabouraud's dextrose agar + yeast medium and attained complete sporulation by 15^{th} day.

Influence of soil pH and pH of the insect cuticle on the survival of soil inhabiting fungi were reported by St leger *et al*. (1998). They studied the impact of pH on the expression of different cuticle degrading enzymes produced by *M. anisopliae* and reported that alkaline pH of the insect cuticle triggered the production of enzyme protease thus making easier to degrade hard surface of the cuticle and allowed penetration of the pathogenic fungi.

In another study by St leger *et al*. (1999), compared the growth characteristics of *M. anisopliae* wild type and mutants in different pH and noticed that the mutants showed reduced growth at pH 8 and failed to produce subtilisin protease (protein digesting enzyme). Namasivayam *et al*. (2015) determined the effect of pH on the colony count of *M. anisopliae* in soil. Maximum fungal colony count of 77.2×10^8 was recorded at pH 7 when compared to that of pH 5.5 (4.2×10^8).

2.2.2. Salinity Tolerance

Rangel *et al*. (2008) elucidated the effect of osmotic stress at the levels of 0.6, 0.8 and 1 M concentration of salt on the conidial germination speed and virulence of *M. anisopliae* isolate ARSEF 25 on insect host, *Tenebrio molitor*. In this study, they observed that *M. anisopliae* was able to grow at higher salt concentration (NaCl or KCl) of 1 M and conidia produced under higher osmotic stress (NaCl at 0.8 M) were found more virulent than conidia produced under control (media without salt). Moreover, heat tolerance of conidia decreased as salt concentration increased, which may be due to the enhanced water permeability.

2.2.3. Temperature Tolerance

Metarhizium are one of the mesophilic fungi that grow at temperatures between 10 and 40°C, with optimal temperature for germination and growth between 25 and 30°C (Roberts and Campbell, 1977).

Ouedraogo *et al*. (1997) compared strains of *M. anisopliae* with *M. flavoviride* for temperature tolerance as the strains of *M. anisopliae* were better adapted to higher temperatures than strains of *M. flavoviride*. Hallsworth and Magan (1999) suggested that the upper limit temperature for mycelial growth of *M. anisopliae* was between 37°C to 40°C. Yewale (2001) reported that the optimum temperature required for the growth and sporulation of *M. anisopliae* was at 25°C. Similarly, Dimbi *et al*. (2004) also assessed the growth and sporulation of six isolates

of *M. anisopliae* and observed that optimum temperature for the growth and sporulation was at 25°C.

Ekesi *et al*. (2003) assessed the influence of soil temperature on the survival and infectivity of four isolates of *M. anisopliae* against tephritid fruit fly puparia and noticed the mortality of puparia at temperatures of 20°C to 30°C caused by all isolates. The results indicated that mortality of puparia caused by *M. anisopliae* had an influence on temperature. According to Chen and Zhu (2004), temperature variations triggered some physiological changes leading to the synthesis of heat shock proteins. Rangel *et al*. (2008) studied the effect of different chemical stresses produced on the conidia of *M. roberstii* and found that conidia produced on the media supplemented with KCl or NaCl were two fold more tolerant to UV-B radiation and heat than conidia produced on PDAY.

According to the study conducted by Rangel *et al*. (2005), *M. anisopliae* isolates from different geographical regions showed a wide variations in temperature tolerance claiming that, isolates collected near equator were more tolerant to higher temperature (40- 50°C) than isolates collected from high altitudes. Nussenbaum *et al*. (2013) conducted a study on *M. anisopliae* strains for their temperature tolerance and classified them based on the optimum temperature for conidial germination as cold active (germinate at 5°C), heat active (germinate at 37°C) and meso-thermo active (unable to germinate beyond these temperatures). Experiment conducted by Teja and Rahman (2016) on screening of four isolates of *M. anisopliae* for temperature tolerance revealed that conidial germination was observed only at 25°C, 30°C and 35°C and no germination at 40°C. Among the different isolates, *M. anisopliae* isolate LaMa1 showed higher radial growth at 35°C compared to other isolates, conveying isolate LaMa1 to be thermotolerant.

Samuels *et al*. (1989) detailed a positive relation between rate of conidial germination and the virulence of entomopathogenic fungi towards target pest in the effect of temperature. Moreover, Dimbi *et al*. (2004) studied the effect of

temperatures on germination, radial growth and virulence of *M. anisopliae* to three species of tephritid fruit flies and found an increase in the infectivity by *M. anisopliae* isolate as the temperature increased till the optimum temperature of that isolate was reached. Another study by Ummidi *et al*. (2013) on *Metarhizium* and *Beauveria*, reported that the strains which germinated faster were more virulent than those with slow germination rate. Faria *et al*. (2015) also conveyed that the rate of conidial germination was a significant virulence determining factor and found to be a reason for shorter survival time of *Spodoptera frugiperda* larvae. The potential of entomopathogenic fungi to infect increased with the increase in temperature till the optimum temperature for that particular isolate was reached.

2.2.4. Drought Tolerance

Studies on screening of isolates to drought tolerance are essential for effective use in biocontrol programme. Chen *et al*. (2014) identified an isolate of *M. anisopliae* MAX-2, exhibiting great potential for growth under dessication stress and showed higher efficacy to *Tenebrio molitor* larvae at below 30 per cent moisture level.

Hallsworth and Magan (1995) physiologically manipulated the conidia of fungal cultures in order to enhance the germination at low water activity. Usually under water stress, germination of conidia and host infection cannot occur. Cultures were grown on SDA with glycerol and trehalose and water availabilities of media were adjusted from 0.99 a_w to 0.93 a_w, to obtain conidia with modified polyol and trehalose. The result showed that conidia with higher intracellular polyol and glycerol germinated quickly and were more pathogenic at low water activity. Similarly, Magan (2001) reported that xerophilic fungi under water-stress conditions were able to synthesize low molecular weight compounds like glycerol and polyols which enable them to survive under extreme conditions of environmental stresses. The capacity to withstand wide range of water activities (a_w) was tested among *B. bassiana*, *M. anisopliae* and *P. farinosus* using agar medium modified with

polyethylene glycol 200 or 400 and 600 with water activities ranging from 0.99 to 0.88. There was no conidial germination observed below 0.92 a_w among the fungi.

A study conducted by Matawele *et al*. (1994) developed more virulent UV-mutant strains of *M. anisopliae* with low water activity, which was effectively controlled green leafhoppers than wild type strains. Borisade and Magan (2014) elaborated the effects of different levels of water activity and temperatures on the growth and sporulation of strains of *M. anisopliae*, *B. bassiana* and *I. farinosa*. Growth on SDA with different water activities modified with glycerol such as 0.99, 0.98, 0.96 and 0.94 a_w was measured at four different temperatures such as 25°C, 30°C, 35°C and 37°C, observed that all the strains of *M. anisopliae* were grown at 25°C to 35°C and 0.99 a_w to 0.96 a_w and at the same time only two strains tolerated extreme water stress at 0.94 a_w.

2.2.5. Insecticide and Fungicide Tolerance

Compatibility of entomopathogenic fungi with pesticides and fungicides is a concern in the integrated pest management. Several studies had demonstrated that *M. anisopliae* was a dominant species in intensively cultivated arable lands and it was thought to be due to the ability of *M. anisopliae* to tolerate agricutltural chemicals and mechanical disturbance (Vanninen, 1995 and Vanninen and Hokkanen, 1988).

Quintela and McCoy (1998) assessed the effect of imidacloprid and two EPF on the survival of *Diaprepes* root weevil grub in the soil and observed that larval mortality had increased synergically when sub-lethal doses of imidacloprid and *M. anisopliae* or *B. bassiana* were applied.

Filho *et al*. (2001) noticed that imidacloprid didn't cause any effects on *M. anisopliae* at maximum dose recommended for the field but shown inhibitory effect at minimum dose while Neves *et al*. (2001) found that there was no toxicity of imidacloprid to *M. anisopliae* and it did not affect the germination and radial growth of *M. anisopliae*. Tanzini (2002) reported the effects of pesticides on

entomopathogens that can vary according to the nature and concentration of chemical products and pathogen species, which resulted in low conidial viability and pathogen virulence to certain pest.

Mochi *et al*. (2005) evaluated the influence of some acaricides, fungicides, insecticides and herbicides on *M. anisopliae* inoculated into the autoclaved soil. The effects of chemicals evaluated were based on the fungal respiratory activity. No remarkable effect of pesticides on *M. anisopliae* were observed except fungicides. Fungicides like copper oxychloride, tebuconazole and chlorothalonyl were more effectiviely influenced the respiratory activity of *M. anisopliae* by lowering CO_2 production. Insecticides like imidacloprid, deltamethrin had no effect on fungus in soil while trichlorfon caused a significant reduction in CO_2 indicating that toxic action of pesticides on *M. anisopliae* in soil is negligible.

Another study of Mochi *et al*. (2006) investigated the effect of pesticides (abamectin, trichlorfon and ametrin) and fungicides (chlorothalonyl and tebuconazole) on the pathogenicity of *M. anisopliae* against *Ceratits capitata* in soil. Fungal suspension were inoculated into glass beaker containing soil samples and fungicides, pesticides were incorporated separately. Then the larvae were accommodated and the results found that fungicide application decreased the pathogenic activity of *M. anisopliae* on *C. capitata* significantly than pesticide application. The pupal survival was reduced and no effect was observed at larval stage. Here, the fungicide application was known to reduce the survival period of *C. capitata*, causing 82.5 to 86.2 per cent mortality compared to control (fungus only) with 95 per cent mortality.

Akbar *et al*. (2012) compared the compatibility of four isolates of *M. anisopliae* to different insecticides and fungicides. Isolate M70 recorded maximum radial growth in PDA amended with spinosad on the tenth day of treatment with diameter of 6.81 cm and spore yield of 1.26×10^8/ml, whereas PDA amended with indoxacarb, imidacloprid, acetamiprid, cypermethrin induced moderate conidial

germination of isolate. In case of fungicides, profenofos, lufenoron, chlorpyrifos and mancozeb caused complete inhibition of conidial germination in all isolates. Among the insecticides and fungicides tested, spinosad, imidacloprid, acetamiprid were more compatible to *Metarhizium* than other insecticides tested and the rest fungicides were not compatible.

2.3. BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae*

Several studies were conducted on various defense mechanisms exhibited by the microorganisms to cope up with extreme environmental conditions. This may include enzymatic activities and biochemical production.

2.3.1 Catalase, Peroxidase and Esterase Activity

Sujatha and Padmaja (2014) evaluated biochemical alterations of two isolates of *M. anisopliae* under thermal stress and noticed a considerable increase in the antioxidant enzymes catalase and peroxidase and slight loss in intracellular enzyme, esterase at 37°C. Antioxidant enzymes enable the fungi to thrive under abiotic stress conditions, by detoxifying H_2O_2 into water and oxygen resulting from relative oxygen species (ROS).

According to the study conducted by Hernandez *et al*. (2010), *M. anisopliae* exhibited an increase in catalase and peroxidase activity during germination and growth accompanied by decreasing germination time and increasing pathogenicity. Miller *et al.* (2004) noticed alterations in isozyme profiles for catalase, peroxidase and superoxidase dismutase in the spores of UV- resistant strains and less resistant strains of *M. anisopliae*. They observed the spores of UV resistant strain demonstrating different isozyme profiles for these enzymes than less resistant strains. Hisada *et al*. (2005) analysed the expression of two catalase genes from *Aspergillus*

oryzae and found that catalases were essential in reducing toxic effects of oxidative stresses that are induced by hydrogen peroxide.

Bilinski (1988) reported induction of catalase in *Streptomyces cerevisiae* when the cells were exposed to temperatures from 22°C to 37°C. A 6-fold increase in peroxidase in *Neurospora crassa* cells had been also described by Machwe *et al*. (2002). Similarly Angelova *et al*. (2005) also reported thermotolerance in *N. crassa* cells and it was assumed that heat shock and oxidative stress stimulated the induction of peroxidase at higher level, resulting thermotolerance.

Padmini and Padmaja (2014) elaborated the defensive role of antioxidant enzymes in *B. bassiana* under abiotic stress conditions. An increase in temperature from 25°C to 33°C, enhanced the production of peroxidase than control. Moreover, enhanced expression of catalase enzyme was also observed under insecticide and fungicide induced stress.

2.3.2. Estimation of Trehalose Content

Trehalose is an essential sugar that protect the cells by preserving the integrity of membranes and prevent damage under stressed environments. Trehalose hydrolysis by trehalase is important in various physiological processes of fungi like spore germination and restoration of resting cells.

Hottiger *et al*. (1987) and Eleutherio *et al*. (1993) reported that cells accumulate trehalose in response to increasing temperature, cold shock, freezing, dehydration, osmotic stress, carbon starvation and stationary-phase growth. It is known to accumulate trehalose upto 30 per cent of dry mass in fungal cells in response to stress conditions like heat shock, water stress and nutritive stress (Kandror *et al*., 2004).

Thevelein (1984) reported that high concentrations of trehalose and polyols in conidia can impart stress tolerance in many entomopathogenic fungi. The fungus, *M. anisopliae* showed relatively small amount of trehalose in all treatments. Jin *et al*.

(2015) suggested that the virulence and *in vivo* growth of *M. acridum* was contributed by the sugar trehalose.

According to Hallsworth and Magan (1994), optimization of trehalose content has increased the survival of conidia of entomopathogens and they had also conducted another study evaluating the effect of variation in temperature and pH in the polyol and trehalose content of entomopathogenic fungi (Hallsworth and Magan, 1996). Growth of EPF was observed at temperatures of 5°C to 35°C and pH ranging from 2.9 to 11.1. Conidia of *M. anisopliae* recorded less than 25 mg trehalose per gram of conidia in all treatments. Amount of polyols and trehalose were higher between 20°C and 30°C and pH between 4 and 8.

Singer and Lindquist (1998) found that trehalose and heat shock proteins act interdependently in increasing thermotolerance in which trehalose helps in stabilizing native proteins and reduces aggregation of denatured proteins under high temperatures.

Rangel *et al*. (2008) analyzed the conidia of *M. anisopliae* produced under four different stress conditions like heat shock, nutritive, osmotic and oxidative stresses. They also tested for the induction of tolerance to these stresses and variations in trehalose content in the conidia. Conidia produced on osmotic and nutritive stress media showed two-fold more tolerance to UV-B radiation and heat. But high accumulation of trehalose was observed in the conidia produced on nutritive stress medium.

2.4. *IN VITRO* EVALUATION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* FOR THEIR BIOCONTROL EFFICACY

Tribolium castaneum is a cosmopolitan and a well known stored-product pest. Loss caused by the pest is enormous and the most frequent control measure adopted is fumigation. The development of resistance due to the repeated application of

insecticides paved a way for the use of entomopathogenic fungi on control of stored-product pest and the most effective among them is *B. bassiana* and *M. anisopliae*.

2.4.2. Biocontrol Efficacy of *Metarhizium anisopliae*

Batta *et al*. (2005) studied the effect of *M. anisopliae* and four types of dust carrirers on mortality, infestation rate and life cycle duration of red flour beetle. Results showed that combinations of dust and fungal conidia caused significant higher mortality ranging from 53.3 to 73.3 per cent when compared to control (dust carrier alone). Wakil *et al*. (2014) reported 73.3 to 86.7 per cent mortality in *Sitophilus oryzae* by *M. anisopliae.*

Biological control of red flour beetle by seven entomopathogenic fungi like *B. bassiana*, *P. farinosus*, two species of *Isaria* and *Lecanicillium* were assessed by Komaki *et al*. (2017). They observed that mortality rate was varied between 34.6 to 100 per cent after 10 days of treatment. Conidial concentrations of 10^5 to 10^7 caused higher mortality rates in adults of red flour beetle by all isolates.

Padin and Vasicek (1997) reported pathogenicity of *B. bassiana* on *adult T. castaneum*. They observed that conidia of 0.5 g per 20 insects out of 0.1 g and 1 g per 20 insects recorded mortality of 87 per cent within 21 days of exposure.

Materials and methods

3. MATERIALS AND METHODS

The study on 'Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin' was carried out in the Department of Agricultural Entomology, College of Horticulture, Vellanikkara during the period 2017- 2019. The materials and methods adopted for various experiments to meet the objectives are given below.

3.1. COLLECTION OF SOIL SAMPLES AND ISOLATION OF *Metarhizium anisopliae*

Purposive soil sampling surveys were conducted in five districts of Kerala *viz*., Thrissur, Palakkad, Ernakulam, Idukki and Alappuzha. The locations were selected based on prevalence of abiotic stresses in each locale. Thrissur district was selected due to high soil acidity, Palakkad was selected as the district experiencing highest temperature in the state, Ernakulam was selected due to its higher soil salinity. Likewise, Alappuzha was selected, reporting acidic as well as saline soil and due to frequent application rates of pesticides and fungicides, Idukki was selected. Locations of survey for soil sample collections are depicted in Table 1, Plate 1.

Five hundred grams of soil sample were collected from each location representing areas of drought, salinity, acidity, areas with high temperature, insecticide and fungicide use following standard protocols. Soil samples were drawn from each location randomly at 15 cm depth using an auger. The samples were pooled after removing dry litter, small stones, root fragments and other debris. They were then serially numbered and allotted codes (soil sample code) based on the location and number of samples. Samples were subsequently air dried for two days at room temperature and then stored at 4ºC in polypropylene covers and used for enumeration of *M. anisopliae*.

Plate 1. Collection of soil samples from different locations of Kerala

Table 1. Locations of soil sample collection from different districts of Kerala

Sl. No.	Districts	Locations	Sample code
1.	Thrissur	Kattoor 1	KTR1
		Kattoor 2	KTR2
		Kattoor 3	KTR 3
		Karalam 4	KLM 4
		Karalam 5	KLM 5
		Karalam 6	KLM 6
		Nadathara 7	NDR 7
		Nadathara 8	NDR 8
2.	Palakkad	Kuthiramulleri1	KMR1
		Kuthiramulleri2	KMR 2
		Kallampatti 3	KPT 3
		Kallampatti 4	KPT 4
		Orassery 5	ORS 5
		Orassery 6	ORS 6
		Orappad 7	ORP 7
		Orappad 8	ORP 8
3.	Ernakulam	Vyttila 1	VYT 1
		Vyttila	VYT 2
		Vyttila 3	VYT 3
		Vyttila 4	VYT 4
		Vyttila 5	VYT 5
		Vyttila 6	VYT 6
		Vyttila 7	VYT 7
		Vyttila 8	VYT 8
4.	Idukki	Punnapara 1	PP 1
		Punnapara 2	PP 2
		Punnapara 3	PP 3
		Punnapara 4	PP 4
		Pampadumpara 5	PDP 5
		Pampadumpara 6	PDP 6
		Pampadumpara 7	PDP 7
		Pampadumpara 8	PDP 8
5.	Alappuzha	Moncompu 1	MC 1
		Moncompu 2	MC 2
		Moncompu 3	MC 3
		Moncompu 4	MC 4

		Moncompu 5	MC 5
		Moncompu 6	MC 6
		Moncompu 7	MC 7
		Moncompu 8	MC 8

3.1.1. Analysis of Physico-Chemical Properties of Soil Samples

The physico-chemical properties of soil such as soil texture, soil temperature, soil moisture content, pH and Electrical Conductivity (EC) were analysed in the laboratory. Temperature of the soil was recorded *in situ* using soil thermometer and soil moisture content was assessed by gravimetric method. Soil samples of 10 g were weighed into the Petri plates and oven dried at 60°C in hot air oven till constant weight was achieved and the weight was calculated as per Reynolds, (1970),

Electrical conductivity of soil samples (measure of soluble salts) was estimated using a digital electrical conductivity meter (Jackson, 1958). Ten gram of soil sample was taken in a 50 ml beaker and 25 ml distilled water was added to it. It was continuously stirred for 30 min using orbitek magnetic stirrer at 260 rpm and was kept for settling. Electrical conductivity of the soil samples was directly read from the conductivity meter.

Soil pH represents the amount of hydrogen ions present in the soil and it constitute physical properties of soil. A digital pH meter was employed to determine the soil reaction of samples as per the procedure described by Jackson (1958).

3.1.2. Isolation of *Metarhizium anisopliae* from Soil

The fungus *M. anisopliae* was isolated from the soil both by serial dilution pour plating method using Veen's selective medium as well as bait trap method using Greater wax moth, *Galleria melonella* (Sowmya, 2017).

3.1.2. a. Isolation of Metarhizium anisopliae Using Serial Dilution and Pour Plate Method

Isolation of *Metarhizium anisopliae* by serial dilution and pour plate method was carried out as described by Parkinson *et al*. (1971). Ten gram of soil was weighed and mixed with 90 ml of sterile water taken in a 250 ml conical flask to yield 10^{-1} dilution. The suspension was agitated vigorously for 15 min in an orbitek shaker at 260 rpm. Aliquot of 1 ml from the mixture was transferred to test tube to which 9 ml of sterile water was added, resulting in 200 fold dilution. It was then diluted serially upto 10^{-4} dilution. One ml of suspension from 10^{-3} and 10^{-4} dilutions was pipetted out separately into the sterile Petri plates and Veen's selective medium was poured and rotated in clockwise and anticlockwise direction for even distribution of the suspension in the media.

3.1.2. b. Isolation of Metarhizium anisopliae Using Bait Trap Method

Metarhizium anisopliae from soil samples was also isolated following bait trap method developed by Zimmermann (1986), using larvae of Greater wax moth, *Galleria mellonella* as an insect bait. About 50 g of soil was taken in a 100 ml beaker and moistened with sterile water. Two live 4^{th} instar larvae were placed in the sample and the beaker was covered with double layered muslin cloth. For each soil sample, three replications were maintained and the larvae were examined six days after treatment. Mycosed larvae were collected and kept separately in Petri plates containing moistened sterile filter paper for the development of fungal growth.

Mycosed larvae with fungal growth was surface sterilized for two min in sodium hypochlorite (4 %) followed by three washings in sterile water under aseptic conditions. The cadavers were then dried by keeping them in sterilized filter paper before they placed in potato dextrose agar medium for the development of fungal growth. The fungi were subcultured in potato dextrose agar (PDA) medium when the growth was observed.

3.1.3. Characterization of *Metarhizium anisopliae* Isolates

Isolates of *M. anisopliae* were identified through visual examination of fungal growth and morphological studies in slide culture. Molecular characterization and phylogenetic analysis (Kernasa *et al*., 2016) of isolates were also employed in order to confirm different isolates of *M. anisopliae*.

3.1.3. a. Cultural Characters

Cultural characters of *M. anisopliae* isolates were studied by visual observation. The isolates were grown in sterile Petri plates containing PDA medium and were incubated at 26 ± 2ºC for 15 days. The distinguishing characters such as colour, pattern and sporulation of the growth were recorded at an interval of 24 h.

3.1.3. b. Morphological Characters

Slide culture method was followed for morphological studies of *M. anisopliae* isolates (Harris, 1986). Microsocopic observations on shape and size of conidia as well as development of phialides, its size were recorded. Photomicrographs of the isolates were also recorded using an ultrascope image analyzer.

3.1.4. Selection of Suitable Cultural Medium for *Metarhizium anisopliae*

For carrying out further studies, attempts were also made to select the most suitable medium for culturing *Metarhizium anisopliae*. Two media such as, Sabauraud's Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) were compared in the study. Five millimeter fungal disc of the isolates was placed on the centre of the medium and was incubated at 26 ± 2ºC for 15 days. The radial growth of the colony developed by the isolates was measured at 15^{th} day.

3.1.5. Preservation of *Metarhizium anisopliae*

Pure cultures of *M. anisopliae* isolates were maintained on PDA slants by periodic subculturing and preserved at 4ºC under refrigerator for carrying out further studies. It was also preserved for longer period by inoculating a five millimeter

mycelial disc into a vial containing 15 per cent dehydrated glycerol (autoclaved) and stored in refrigerator at 4°C.

3.2. *IN VITRO* SCREENING OF *Metarhizium anisopliae* ISOLATES FOR STRESS TOLERANCE

The effect of abiotic factors like temperature, pH, salinity, drought, insecticide and fungicides on the growth of *M. anisopliae* isolates were studied under *in vitro* conditions. Observations on radial growth or mycelial weight and sporulation (visual) of the isolates at different stress levels were recorded.

3.2.1. Screening of *Metarhizium anisopliae* Isolates for Acidity Tolerance

Acidity tolerance of different isolates of *M. anisopliae* was evaluated as per the protocol of Reetha *et al*. (2014). Hundred milliliters of Potato Dextrose Broth (PDB) taken in each bottle were adjusted to different pH levels of 2.5, 3, 3.5, 4.5, 5.5 using 0.1 N HCl and 0.1 N NaOH and control (pH of PDB= 6) were autoclaved for 20 min. A five millimeter mycelial disc from mother culture of each *M. anisopliae* isolate was cut out using a cork borer and inoculated on to sterilized 100 ml pH adjusted potato dextrose broths. The inoculated broths were kept for incubation at 26 ± 2ºC for ten days after which, fresh mycelial mat was filtered using Whatman No.1 filter paper and the weight was recorded in grams.

3.2.2. Screening of *Metarhizium anisopliae* Isolates for Salinity Tolerance

Tolerance of different isolates of *M. anisopliae* to saline conditions was studied as per the protocol of Amalraj *et al*. (2010). Sodium chloride concentrations of 0.5 M, 1 M, 1.5 M and 2 M were used to study the effect of salinity on the growth of isolates. A five millimeter mycelial disc of each isolate was placed onto the centre of PDA medium containing different concentration of NaCl. Control plates containing PDA without NaCl were also maintained. The plates were incubated at 26

± 2º C for 14 days. Radial growth was measured and the percent reduction of growth was worked out as per Vincent (1927)

Per cent inhibition = (C-T) × 100/C where,

C = Radial growth of isolate in PDA plate (cm)

T = Radial growth of the salt amended PDA plate (cm)

3.2.3. Screening of *Metarhizium anisopliae* Isolates for Temperature Tolerance

Temperature tolerance of *M. anisopliae* isolates was examined as per the protocol of Reetha *et al*., (2014). A five millimeter mycelial disc of each isolate was inoculated onto the sterilized potato dextrose broth of 100 ml in bottle and incubated at different temperatures of 25, 30, 35, 37 and 40°C in a water bath for ten days. Separate control was also maintained at 27°C. After ten days of incubation, the fresh mycelial weight of each isolate was recorded as per 3.2.1.

3.2.4. Screening of *Metarhizium anisopliae* Isolates for Drought Tolerance

Tolerance of *M. anisopliae* isolates to drought was estimated as per the protocol of Amalraj *et al*., (2010). Polyethylene glycol (PEG 6000 Da) at different concentrations of 10, 20, 30 and 35 per cent representing osmotic potential of -0.15, -0.49, -1.03 and -1.20 M Pa was added into potato dextrose broth of 100 ml taken in separate bottles and the control (without PEG) were autoclaved for 20 min. A five millimeter mycelial disc of each isolate was inoculated onto the sterilized broth and incubated at 26 ± 2°C for ten days. The fresh mycelial weight of each isolate was measured at tenth day as per 3.2.1.

3.2.5. Screening of *Metarhizium anisopliae* Isolates for Insecticide and Fungicide Tolerance

Metarhizium anisopliae isolates were tested for its tolerance to selected insecticides and fungicides as per the protocol of Grover and Moore (1962) following

the method of poisoned food technique. Four insecticides *viz*., spinosad, cypermethrin, imidacloprid and chlorantraniliprole as well as three fungicides namely copper oxychloride, carbendazim and hexaconazole were used in this experiment (Table 2). Compatibility of the isolates was evaluated by exposing them to different doses *i.e*., lower dose, recommended dose and higher dose.

Table 2. Details of insecticides and fungicides used in the study

Chemical name	Trade name and formulation	a.i (g/ha)	Doses used (ml or g/l)		
			Lower dose	Recommended dose	Higher dose
Spinosad	Taffin, 45 SC	100	0.28	0.33	0.38
Cypermethrin	Cyperguard, 25 EC	40	0.35	0.40	0.45
Imidacloprid	Admire, 70 WG	20	0.05	0.10	0.15
Chlorantraniliprole	Coragen, 18.5 SC	30	0.25	0.30	0.35
Copper oxychloride	Fytolan, 50 WP	500	0.20	0.25	0.30
Carbendazim	Bavistin, 50 WP	250	0.50	1.00	1.50
Hexaconazole	Contaf, 5 EC	35	1.50	2.00	2.50

Desired quantity of chemical was measured out and mixed thoroughly with the sterilized PDA medium and poured onto the Petri plates. PDA medium without fungicides and insecticides served as control. A five millimeter mycelial disc of each isolate was placed at center of the medium and was kept for incubation at 26 ± 2°C for 14 days. Radial growth was measured and the per cent inhibition was calculated as per 3.2.2.

3.3. BIOCHEMICAL CHARACTERIZATION OF *Metarhizium anisopliae* ISOLATES

Promising *M. anisopliae* isolates identified during *in vitro* screening were subjected to biochemical analysis for elucidating the stress tolerance mechanisms involved. Antioxidant enzymes like catalase and peroxidase that impart tolerance to various stresses and intracellular enzyme esterase were assayed. A disaccharide,

trehalose which is a source of energy and a biomolecule that help survival under freezing and dehydration was quantified. In the study, a temperature tolerant and a drought tolerant isolate obtained as per the experiment 3.2.3 and 3.2.4 were subjected to analyze the variation in the enzymatic activity and quantity of trehalose with respect to stress.

3.3.1. Sample Preparation for Total Protein and Enzyme Assay

For total protein, esterase, catalase and peroxidase assay, fungal extracts were prepared as described by Sujatha and Padmaja (2014) with slight modifications. In the study, temperature tolerant isolate MC 7 was grown in a PDB medium for eight days at 35°C and 37°C. Similarly, drought tolerant isolate MC 2 was also allowed to grow at different concentrations of PEG (20 % and 35 %) amended in PDB broth. Control was maintained by keeping MC 7 at 27ºC and MC 2 without adding PEG in PDB. After eight days of incubation, mycelial mats were taken, washed thoroughly with sterile water and stored in refrigerated condition for carrying out different biochemical analysis.

To carry out protein and esterase assay, 4.5 g of fungal mycelial mat of each isolate was ground with 10 ml of sodium phosphate buffer (pH 7.4) in pestle and mortar. The solution was sonicated for 8 min and then centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant obtained was stored in deep freezer (-18ºC) at required aliquots until estimation.

In case of catalase and peroxidase estimation, 4.5 g of mycelial mat of each isolate was ground with 10 ml of ice cold sodium phosphate buffer (pH 7) in precooled pestle and mortar. It was then sonicated for 8 to 10 min and centrifuged at 18,000 rpm for 15 min in case of peroxidase assay and 10,000 rpm for 10 min at 4°C for catalase assay. The supernatants were immediately used for the estimation within 24 h period.

3.3.2. Total Protein Assay

Total protein content of fungi was estimated as per the method of Lowry *et al*. (1951).

3.3.2.1. Preparation of Standard Bovine Serum Albumin Solution and Reagents

Stock solution was prepared with bovine serum albumin by taking 50 mg BSA in 50 ml of distilled water. From this, working standard solution was prepared by pipetting out 10 ml of stock solution and making up to 50 ml with distilled water, which implies that 1 ml of solution contains 200 µg protein. Aliquots of 200 µl, 400 µl, 600 µl, 800 µl and 1000 µl were pipetted out into different test tubes and made upto 1 ml with distilled water. A blank was kept with distilled water alone and the four reagents required in the assay were prepared as follows,

Reagent A : Sodium carbonate (2 %) dissolved in NaOH (0.1 N)

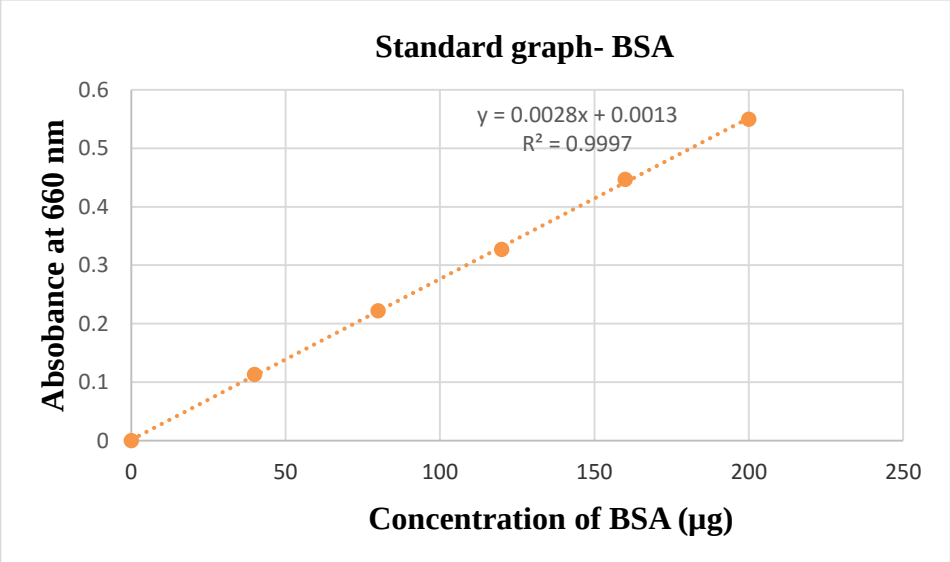
Reagent B : Copper sulphate solution (0.5 %) dissolved in sodium potassium tartarate solution (1 %)

Reagent C : Mixture of reagent A, 50 ml and reagent B, 1 ml, freshly prepared

Reagent D: Folin-ciocalteu reagent and distilled water taken in 1:1 ratio, freshly prepared

3.3.2.2. Preparation of Standard Graph using BSA Solution

Five milliliter of Reagent C was added to all test tubes including blank. Each aliquot was then mixed well and kept for 10 min. After 10 min, 0.5 ml of reagent D was added and incubated in the dark at room temperature for 30 min. The contents developed a blue colour and corresponding absorbance reading was recorded at 660 nm using Agilent Cary 60 UV Vis spectrophotometer. Standard graph was drawn with obtained OD value corresponding to BSA concentrations.



3.3.2.3. Total Protein Content of Metarhizium anisopliae Isolates

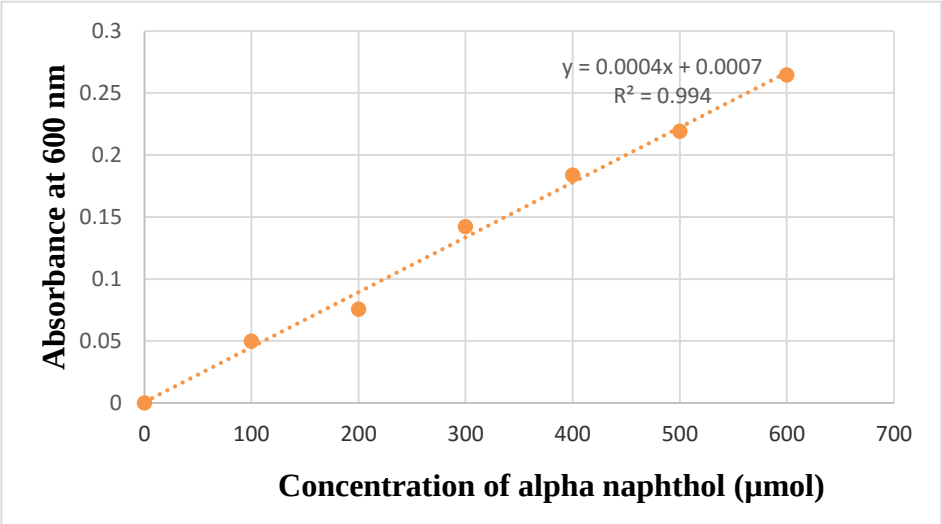
Fifty microliter supernatant was taken in a test tube to which 2.5 ml of reagent C was added and mixed well. The mixture was incubated for 10 min and 0.25 ml of reagent D was added and again incubated for 30 min in the dark at room temperature. The absorbance reading was taken at 660 nm using Agilent Cary 60 UV Vis spectrophotometer. The protein content obtained from the standard graph was expressed in mg/ml.

3.3.3. Esterase Assay

Esterase activity of *M. anisopliae* isolates was carried out as per the procedure of van Asperen (1962).

3.3.3.1. Preparation of Standard Graph using Alpha Naphthol

Alpha naphthol stock solution of 10 mM was prepared by dissolving 0.036 g alpha naphthyl acetate in 25 ml methanol. Aliquots such as, 10 µl, 20 µl, 30 µl, 40 µl, 50 µl and 60 µl of the working standard solution were taken in test tubes and made up to 1 ml using methanol.



Two milliliters of sodium phosphate buffer (pH 7.4) was added to each test tube and 1 ml of sodium phosphate buffer alone was kept as blank. The contents were incubated for 10 min at 30°C with constant stirring followed by the addition of 0.05 ml dye solution containing 22.5 mg fast blue RR salt in 2.25 ml of distilled water and SDS (5 %). The mixture was then incubated for 10 min at 37°C. A reddish brown colour developed which was read at 600 nm using Agilent Cary 60 UV Vis spectrophotometer. Standard graph was prepared using OD values obtained corresponding to alpha naphthol concentrations.

3.3.3.2. Esterase Activity of Metarhizium anisopliae Isolates

Fifty microliter supernatant was taken in each test tube and one milliliter of 30 mM alpha naphthyl acetate (0.028 g alpha naphthyl acetate dissolved in 5 ml acetone) was added and kept for incubation at 30°C for 10 min. Dye solution (0.05 ml) was added after 10 min and again the mixture was incubated for 10 min at 37°C. The absorbance reading was noted at 600 nm in Agilent Cary 60 UV Vis spectrophotometer. The esterase activity was expressed in enzyme units per mg of protein per minute which was obtained from the standard graph using alpha naphthol.

3.3.4. Catalase Activity of *Metarhizium anisopliae* Isolates

Catalase activity of *M. anisopliae* isolates was estimated as per the procedure of Sadasivam and Manickam (2008). Fifty microliter of supernatant was taken in a cuvette and 3 ml of H_2O_2-phosphate buffer (0.16 ml of H_2O_2 diluted to 100 ml with phosphate buffer of pH 7) was added and mixed well using a glass rod. Control was kept by mixing supernatant with H_2O_2 free phosphate buffer. Absorbance was noted at 30 sec interval for 5 min at 240 nm using Agilent Cary 60 UV Vis spectrophotometer. Catalase activity was expressed in terms of change in absorbance per minute per mg of protein.

3.3.5. Peroxidase Activity of *Metarhizium anisopliae* Isolates

Peroxidase activity of *M. anisopliae* was determined as per the protocol of Mahadevan and Sridhar (1986). Two milliliters sodium phosphate buffer, 1 ml of 20 mM guaiacol and 50 µl of supernatant were taken in a clean dry cuvette and was placed in an Agilent Cary 60 UV Vis spectrophotometer. Fifty microliter of hydrogen peroxide (10 mM) was added finally to the cuvette and the absorbance was noted immediately at an interval of 30 sec for 5 min at 470 nm. Change in absorbance per min was noted and used to calculate the peroxidase activity. It was expressed as change in absorbance per minute per tissue weight.

$$\text{Peroxidase activity} = \frac{\text{Difference in OD value per min}}{\text{Volume of enzyme extract (ml)}} \times \frac{\text{Total volume of enzyme assay (ml)}}{\text{Weight of mycelia (g)}}$$

3.3.6. Trehalose Content

Amount of trehalose present in the mycelia of *M. anisopliae* isolates at different temperatures and drought levels were assessed as per anthrone- sulphuric acid colorimetric method of Wang *et al*. (2013).

3.3.6.1. Preparation of Standard Graph Using Trehalose

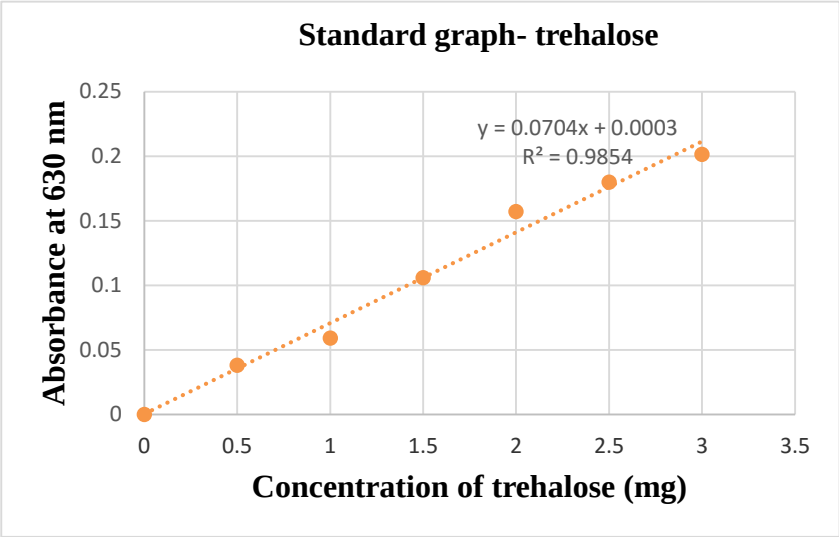
One gram of trehalose was dissolved in 1000 ml of distilled water and Six volumes of stock solution *i.e*., 0.5, 1, 1.5, 2, 2.5 and 3 ml were pipetted out into the test tubes. Five milliliters freshly prepared anthrone sulphuric acid (0.1 g of anthrone dissolved in 95 per cent sulphuric acid) and 4 ml of trichloroacetic acid were added to each aliquot and they were reacted in a boiling water bath for 10 min. The mixture was allowed to cool at room temperature and the absorbance of yellow colour developed was recorded at 630 nm using Agilent Cary 60 UV Vis spectrophotometer. Standard graph was prepared using readings obtained corresponding to the concentrations of trehalose.

3.3.6.2. Sample Preparation for Trehalose Assay

Fungal mycelia of temperature and drought tolerant isolates were prepared as described in 3.3.1. Approximately 1 g of mycelial mat along with broth (3 ml) was ground using a pestle and mortar. Supernatant (2ml) was leached with 3 ml of ice cold sterile distilled water through centrifuging at 5000 rpm for 5 min and the washing was repeated twice. The pellet formed was then mixed with 4 ml trichloroacetic acid and centrifuged for 20 min at 10,000 rpm and the supernatant was stored in a 15 ml centrifuge tube. The reaction was repeated thrice and the supernatant conserved were pooled together and made upto 50 ml using trichloroacetic acid.

3.3.6.3. Trehalose Content of Stress Tolerant Isolates

From the mycelial extract made up as described above, an aliquot of 0.5 ml enzyme extract was mixed with 5 ml of anthrone sulphuric acid and 4 ml of trichloroacetic acid. The mixture was heated in a water bath, then cooled and absorbance was read as previously described. Trehalose content of the mycelia was calculated from the standard graph and expressed in milligram of trehalose per min per gram of mycelia.



3.4. *IN VITRO* EVALUATION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* FOR THEIR BIOCONTROL EFFICACY

Metarhizium anisopliae isolates identified as tolerant to various abiotic stresses were evaluated for their bioefficacy against active stages (17- 19 day old adults and 5th instar grubs) of red flour beetle, *Tribolium castaneum* as per the protocol of Finney (1971). Experiment was conducted with the stress tolerant isolates of *M. anisopliae* obtained from different locations (MC 2, MC 4 and MC 7), positive control (NBAIR strain of *M. anisopliae*), negative control (without *M. anisopliae*) and the insect. The details of the design are presented below.

Details of the design used in the experiment

Design	**Probit analysis**
Concentrations	5
No. of insects/ replication	10
Stage of insect	5th instar grubs 17-19 day old adults
Replication	3
No. of isolates	**4**

3.4.1. Preparation of Fungal Culture and Spore Suspension of *Metarhizium anisopliae*

Isolates preserved in 15 per cent glycerol as previously described in 3.1.4 were pure cultured in PDA medium at room temperature. Fourteen day old culture of each isolate was taken aseptically by scraping the spores into a sterile test tube containing sterile water in order to prepare the spore suspensions. A drop of 0.1 per cent tween 80 was added to it and the suspension was vortexed for 2 min so that the spores get dispersed uniformly. The suspension was then filtered through a sterilized double layered muslin cloth and spore count of the suspensions was enumerated using a Neubauer haemocytometer (Lomer and Lomer 1996) as follows,

$$\text{Number of spores} = \frac{X \times 400 \times 10 \times 1000 \times D}{Y}$$

Where, X= Average number of spores per small square

D= Dilution factor

Y= Number of small squares counted

400= Total number of small squares

10= Depth factor

1000= Conversion factor from mm^3 to cm^3

The suspensions of all isolates were adjusted to 10^9 spores per ml and it was then serially diluted to have lower concentrations of isolates *i.e.*, 10^8, 10^7, 10^6 and 10^5 spores per ml of suspension respectively.

3.4.2. Rearing of Test Insect, *Tribolium castaneum*

The test insect was reared in the laboratory at 30 ± 2ºC and 80 ± 5 per cent relative humidity as per the protocol of Bhatia and Pradhan (1968). Adults of *T. castaneum* (15 to 20) were released into plastic containers containing 250 g of sterilized wheat flour enriched with five per cent brewer's yeast. The containers were

then covered with muslin cloth and placed in a culture room and it was then protected with ant well. After five days of oviposition period, the adults released were sieved out and transferred to fresh rearing containers. The process was repeated in order to get continuous supply of adults and grubs of known age. Adults of 17 ± 2 day old and 4^{th} or 5^{th} instar grubs were used for the bioassay.

3.4.3. Bioassay

Ten numbers of insects were released into each Petri plate. Respective spore suspensions of isolates (1 ml) was poured uniformly on the insects in the sterile Petri plates containing sterile tissue paper. For grubs, wheat flour was provided after 24 h treatment. The experiment was laid out following probit analysis under laboratory condition with three replications and control was kept using sterile water. Number of insects killed was observed at 24 h interval upto 15 days and the percentage mortality was calculated after making necessary corrections using Abbott's formula (Abbott, 1925). Probit analysis was carried out using the soft ware, PoloPC to determine dose-mortality relationship, LT_{50} (Lethal time taken to kill 50 per cent of test populations), LT_{90}, fiducial limits and other parameters.

Statistical Analysis

Data was accorded to Analysis of Variance (ANOVA) employing Web Agri Stat Package (WASP 2.0). Multiple comparison between the treatment means were done with Duncan's Multiple Range Test (DMRT) and appropriate transformations were considered according to the method elucidated by Gomez and Gomez (1984).

Results

4. RESULTS

The study on "Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin" was conducted at College of Horticulture, Vellanikkara, to identify stress tolerant isolates of *M. anisopliae* through *in vitro* screening and also to evaluate its biocontrol potential against *Tribolium castaneum*. The results of the experiments are presented in this chapter.

4.1. COLLECTION OF SOIL SAMPLES AND ISOLATION OF *Metarhizium anisopliae* ISOLATES

Purposive soil sampling surveys were conducted from different stressed locations of Kerala *viz*., Thrissur, Palakkad, Ernakulam, Idukki and Alappuzha and a total of 40 soil samples was collected from five districts at the rate of eight soil samples from each district.

4.1.1. Analysis of Physico-Chemical Properties of Soil Samples

The physico-chemical properties of soils such as soil temperature, soil texture, soil moisture content, soil pH and EC were recorded and observations are depicted in Table 3.

Among 40 soil samples collected, sample code KTR 1 to KTR 3, KLM 4 to KLM 6, NDT 7 and NDT 8 represents the soil samples of Thrissur district. Palakkad soil samples were assigned with codes KMR 1 and KMR 2, KPT 3 and KPT 4, ORS 5 and ORS 6 and ORP 7 and 8. Codes VYT 1 to VYT 8 represents soil samples of Ernakulam district, MC 1 to MC 8 represents Alappuzha soil samples and PP 1 to PP 4, PDP 5 to PDP 8 represents soil samples of Idukki district.

The temperature of soils from 40 different locations ranged from 29 to 46ºC while the highest temperature of 46ºC was recorded from Palakkad district (Kallampatti 4) and the lowest temperature of 29ºC was recorded from Punnapara 3

and 4 of Idukki district. Among the five districts, Palakkad soils had the highest soil temperature ranging from 43 to 46ºC, followed by Thrissur soils with soil temperature ranging from 40.00 to 43.08ºC. Soils of Idukki district had the lowest temperature of 29.00 to 31.50ºC

Similarly, the soil moisture content of soil samples varied between 1.10 per cent recorded from the location Kallampatti 3 of Palakkad district to 40 per cent from Moncompu 4 of Alappuzha district. Lowest soil moisture content was observed in the soil samples from Palakkad district (1.10 to 2.70 %) while Alappuzha district had the highest soil moisture content, ranging from 34.50 to 40.00 per cent.

Soil pH of the 40 soil samples ranged between 3.40 and 6.00. The lowest pH was recorded from the sample Moncompu 1 of Alappuzha district and the highest from the sample Kuthiramulleri 2 of Palakkad district. Soil samples from different locations were also analysed for electrical conductivity and expressed in dS m^{-1}. Samples from Thrissur and Palakkad districts showed EC ranging from 0.01 to 0.02 dS m^{-1} and highest EC value of 6.68 dS m^{-1} was recorded from the soil sample Moncompu 1 of Alappuzha district.

In Thrissur district, temperature of soil samples collected from eight kole lands varied between 40.00ºC and 43.08ºC with a highest value recorded from the location Kattoor 3 and the lowest from Kattoor 2 and Nadathara 7. Moisture content was varied between 1.20 and 4.00 per cent, where the higher per cent moisture was estimated from the location Karalam 4 and the lowest from Kattoor 1. Soil texture was clay, with pH ranging from 4.40 to 5.40. Soil collected from Kattoor 1 recorded the highest pH value of 5.40 and the lowest pH (4.40) from Karalam. Electrical conductivity estimated were ranged between 0.01 and 0.02 dS m^{-1}.

Likewise, eight locations of Palakkad district, recorded soil temperatures ranging from 44 to 46ºC and soil moisture content of range from 1.10 to 2.70 per cent. Highest moisture per cent of 2.70 was estimated from the sample Kuthiramulleri 2 and

lowest from Kallampatti 3. Soils were sandy clay loam in texture with soil pH ranging from 5.20 to 6.00 and the electrical conductivity ranged from 0.01 to 0.02 dS m^{-1}.

Eight locations of Ernakulam district recorded soil temperature within a range of 36.00 and 37.70ºC where, the highest value was noticed from the sample Vyttila 7 and lowest from Vytilla 3. Soil texture was clay loam and the soil moisture content varied from 23.30 to 28.30 per cent. Soil pH, generally noticed to be acidic and the values were between 4.20 and 4.61, the highest pH 4.61 was reported from the location Vyttila 8 and lowest pH of 4.20 from the locations Vyttila 3 and Vyttila 4. Soil samples had an EC ranging between 4.10 and 4.40 dS m^{-1} with highest EC from the location Vyttila 7 and the lowest from Vyttila 1 and Vyttila 2.

Soil temperature from eight locations of Alappuzha district was between 37.70 and 38.50ºC. Highest soil temperature was recorded from the location Moncompu 1 and the lowest from Moncompu 3. Soil texture was sandy clay with moisture content varying from 34.50 to 40 per cent. Location Moncompu 3 had the highest moisture content (40 %) and lowest in location Moncompu 4. Soil reaction was found to be strongly acidic and the values ranged between 3.45 and 3.90, with the highest being in location Moncompu 5 and the lowest in location Moncompu 8. Electrical conductivity of soil samples ranged from 5.22 to 6.68 indicating slightly saline in nature.

Soil temperature of Idukki district (8 locations) was very low when compared to other districts and the values ranged between 29.00 and 31.50ºC. Highest soil temperature of 31.50ºC was recorded in location Pampadumpara 5 and lowest in Punnapara 3 and Punnapara 4 (29ºC). The soil moisture content was varied between 5.90 and 7.70 per cent, with the highest in the location Punnapara 4 and the lowest in Pampadumpara 5. The soil texture was sandy clay loam except for the location Punnapara 3, 4 and Pampadumpara 8 with clay loamy texture. Soil pH was varied between 4.10 and 5.81, the highest value being from location Pampadumpara 7 and the lowest from Punnapara 2. The electrical conductivity of soil samples was also

low, ranging between 0.41 and 0.91 dS m^{-1} making non-saline in nature. The highest EC (0.91 dSm^{-1}) was recorded from the location Pampadumpara 8 and the lowest from Punnapara 4 (0.41 dSm^{-1}).

4.1.2. Isolation and Enumeration of *Metarhizium* spp.

Metarhizium spp. were isolated from collected soil samples through serial dilution plating method using Veen's selective medium as well as by bait trap method (Tables 4 and 5) [Plate 2].

Among the 40 soil samples collected from five districts, *Metarhizium* spp. were obtained only from five locations of Alappuzha district and these isolates were named as MC 2, MC 3, MC 4, MC 7 and MC 8 corresponding to the location from where the sample was collected.

Metarhizium populations from locations MC 2, MC 3, MC 4, MC 7 and MC 8 (soil samples) ranged from 13.33 to 19.66 × 10^3cfu g^{-1} and 5 to 7.33 × 10^4 cfu g^{-1}. Soil sample of location Moncompu 7 (MC 7) had the highest number of colonies of *Metarhizium* spp. with 19.66 × 10^3 cfu g^{-1} and 7.33 × 10^4 cfu g^{-1} in the Veen's selective medium and the lowest from the location Moncompu 2 (MC 2) with 13.33 × 10^3 cfu g^{-1} and 4.66 × 10^4 cfu g^{-1}.

Mycosis of *Galleria melonella* by bait trap method due to *Metarhizium* infection was also observed in the soil samples from locations MC 2, MC 3, MC 4, MC 7 and MC 8 of Alappuzha district.

4.1.3. Characterization of *Metarhizium* spp.

Cultural, morphological and molecular characterization of the *Metarhizium* isolates obtained during survey were conducted.

Based on the morphological, cultural and molecular characterization, species was confirmed as *Metarhizium anisopliae* and out of five isolates; two isolates such as MC 3 and MC 8 were shown similarity to the isolates MC 2 and MC 4 respectively

Table 3. Physico-chemical properties of soil samples collected from different locations of Kerala

Sl. No.	Location	Soil temperature (°C)	Soil texture	Soil moisture content (%)	pH	EC (dSm^{-1})
1.	**Thrissur** Kattoor 1 (KTR 1)	42.00	Clay	1.20	5.40	0.02
2.	Kattoor 2 (KTR 2)	40.00	Clay	3.90	5.20	0.02
3.	Kattoor 3 (KTR 3)	43.08	Clay	2.90	5.00	0.02
4.	Karalam 4 (KLM 4)	42.20	Clay	4.00	4.40	0.01
5.	Karalam 5 (KLM 5)	41.00	Clay	2.20	4.40	0.01
6.	Karalam 6 (KLM 6)	42.10	Clay	2.80	4.40	0.01
7.	Nadathara 7 (NDT 7)	40.00	Clay	1.20	5.00	0.02
8.	Nadathara 8 (NDT 8)	40.50	Clay	1.60	5.28	0.02
9.	**Palakkad** Kuthiramulleri 1 (KMR 1)	43.00	Sandy clay loam	2.30	5.80	0.02
10.	Kuthiramulleri 2 (KMR 2)	44.42	Sandy clay loam	2.70	6.00	0.02
11.	Kallampatti 3 (KPT 3)	44.10	Sandy clay loam	1.10	5.90	0.02
12.	Kallampatti 4 (KPT 4)	46.00	Sandy clay loam	2.00	5.60	0.02
13.	Orassery 5 (ORS 5)	45.40	Sandy clay loam	2.40	5.60	0.01
14.	Orassery 6 (ORS 6)	44.00	Sandy clay loam	1.88	5.60	0.02
15.	Orappad 7 (ORP 7)	44.70	Sandy clay loam	1.75	5.80	0.02
16.	Orappad 8 (ORP 8)	44.20	Sandy clay loam	1.62	5.20	0.02
17.	**Ernakulam** Vyttila 1 (VYT 1)	37.60	Clay loam	23.30	4.30	4.10
18.	Vyttila 2 (VYT 2)	36.50	Clay loam	25.60	4.50	4.10

19.	Vyttila 3 (VYT 3)	36.00	Clay loam	24.80	4.20	4.30
20.	Vyttila 4 (VYT 4)	37.30	Clay loam	26.60	4.20	4.30
21.	Vyttila 5 (VYT 5)	37.10	Clay loam	28.10	4.60	4.20
22.	Vyttila 6 (VYT 6)	36.20	Clay loam	24.00	4.25	4.38
23.	Vyttila 7 (VYT 7)	37.70	Clay	26.60	4.28	4.40
24.	Vyttila 8 (VYT 8)	37.00	Clay	28.30	4.61	4.21
25.	**Idukki** Punnapara 1 (PP 1)	29.50	Sandy clay loam	7.60	4.90	0.45
26.	Punnapara 2 (PP 2)	31.30	Sandy clay loam	6.50	5.81	0.72
27.	Punnapara 3 (PP 3)	29.00	Clay loam	6.30	5.20	0.70
28.	Punnapara 4 (PP 4)	29.00	Clay loam	7.70	4.74	0.41
29.	Pampadumpara 5 (PDP 5)	31.50	Sandy clay loam	5.90	5.30	0.91
30.	Pampadumpara 6 (PDP 6)	30.60	Sandy clay loam	6.50	4.50	0.69
31.	Pampadumpara 7 (PDP 7)	29.20	Sandy clay loam	6.20	4.10	0.73
32.	Pampadumpara 8 (PDP 8)	30.10	Clay loam	6.00	5.60	0.87
33.	**Alappuzha** Moncompu 1 (MC 1)	38.50	Sandy clay	35.01	3.40	6.68
34.	Moncompu 2 (MC 2)	37.90	Sandy clay	35.00	3.50	5.26
35.	Moncompu 3 (MC 3)	37.70	Sandy clay	34.50	3.80	5.50
36.	Moncompu 4 (MC 4)	38.10	Sandy clay	40.00	3.60	5.50
37.	Moncompu 5 (MC 5)	37.80	Sandy clay	39.00	3.90	5.40
38.	Moncompu 6 (MC 6)	38.00	Sandy clay	36.60	3.50	5.22
39.	Moncompu 7 (MC 7)	38.20	Sandy clay	35.80	3.50	5.26
40.	Moncompu 8 (MC 8)	38.00	Sandy clay	35.20	3.45	6.62

(i) Serial dilution and pour plating

(ii) Bait trap method

Plate 2. Enumeration of *Metarhizium* spp.

Table 4. Enumeration of *Metarhizium* spp. in Veen's selective medium

Sl. No.	Districts	Population of *Metarhizium* (cfu g^{-1}) in Veen's selective medium *	
		$\times 10^3$	**$\times 10^4$**
1.	Thrissur	0.00	0.00
2.	Palakkad	0.00^f	0.00
3.	Ernakulam	0.00	0.00
4.	Alappuzha		
Locations	Moncompu 2 (MC 2)	13.33	4.66
	Moncompu 3 (MC 3)	18.00	5.66
	Moncompu 4 (MC 4)	16.33	5.00
	Mancompu 7 (MC 7)	19.66	7.33
	Moncompu 8 (MC 8)	14.00	5.55
5.	Idukki	0.00	0.00

* Mean of three replication

No. of samples per district = 8

Table 5. Presence of mycosed larva in different soil samples

Sl. No.		Districts	Mycosed larva
1.		Thrissur	-
2.		Palakkad	-
3.		Ernakulam	-
		Alappuzha	
Locations	4.	Moncompu 2 (MC 2)	+
	5.	Moncompu 3 (MC 3)	+
	6.	Moncompu 4 (MC 4)	+
	7.	Moncompu 7 (MC 7)	+
	8.	Moncompu 8 (MC 8)	+
9.		Idukki	-

+: Present, -: Absent
No. of samples per district = 8

and hence further studies were continued with MC 2, MC 4 and MC 7. The important features of each isolates is given below (Table 6) [Plate 3].

4.1.3.1. Metarhizium anisopliae Isolates from Alappuzha

a) MC 2

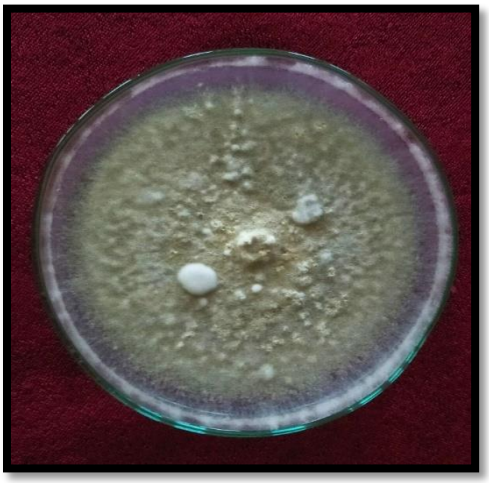
Metarhizium anisopliae obtained from the locations Moncompu 2 and Moncompu 3 were represented as isolate MC 2 and its fungal growth was characterized by initial fluffy white mycelial growth which later turned off-white colour with light green sporulation. The sporulation was observed on the eighth day of incubation and attained complete mycelial growth in the PDA medium by 15^{th} day with creamy colouration on the reverse side of the plate. Conidia were cylindrical dome shaped with a mean length of 3.53 µm and a mean width of 1.61 µm. They were arranged in chains from the tip of phialide. Mean length and width of the phialide were 6.61 µm and 1.51 µm respectively.

b) MC 4

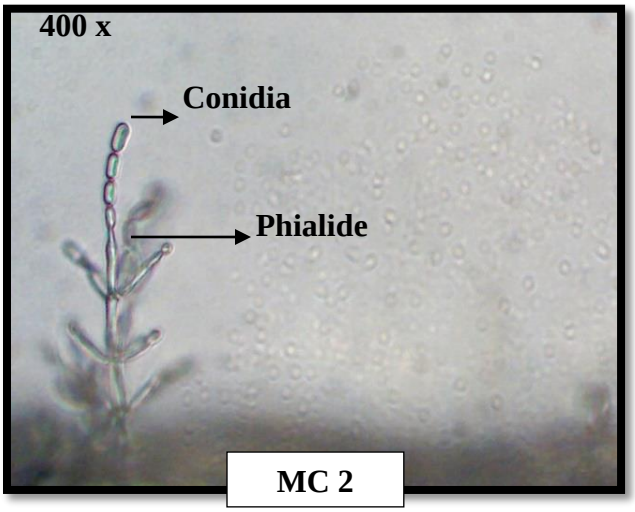
MC 4 isolate obtained from Moncompu 4 and Moncompu 8 locations was observed as white mycelial growth which later turned to green colour with green spores. It began to sporulate on the seventh day of incubation and the growth was completed by 15^{th} day in PDA medium. Reverse side of the plate appeared creamy in colour. Conidia were cylindrical dome shaped with a mean length of 3.72 µm and mean width of 1.38 µm. It formed chains as well as clusters of conidia from the tip of phialide. Mean length of the phialide was 4.72 µm and mean width was 1.32 µm.

c) MC 7

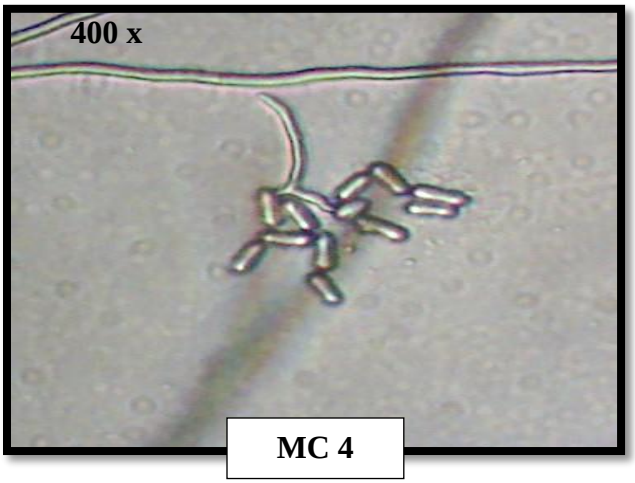
Isolate MC 7 obtained from location Moncompu 7 showed a typical sparse white coloured mycelial growth in PDA medium initially, which later turned to green with dark green sporulation. Growth was completed by 15^{th} day and the reverse side of the plate displayed creamy colour. Conidia were cylindrical dome shaped with a



MC 2



MC 4



MC 7

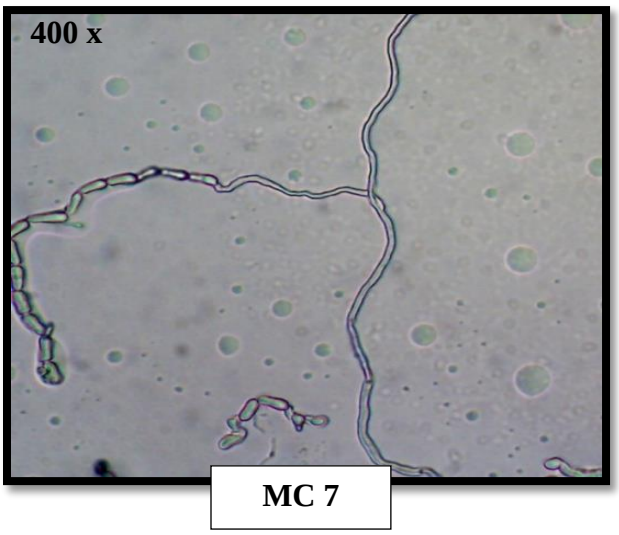


Plate 3. Cultural characters and photomicrographs of *Metarhizium anisopliae* isolates

mean length of 4.05 µm and mean width of 1.70 µm, attached to tip of phialide as chains. The phialide mean length was 7.01 µm and mean width was 1.50 µm.

4.1.3.2. Molecular Characterization of Metarhizium anisopliae Isolates

Molecular characterization of *M. anisopliae* isolates obtained was carried out through ITS sequencing at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The sequences were then blasted in the online BLASTn programme of NCBI in order to find the nucleotide homology of each isolate (Table 7 and 8) [Plate 4].

4.1.3.2.1. Sequence Comparison of the Isolate MC 2

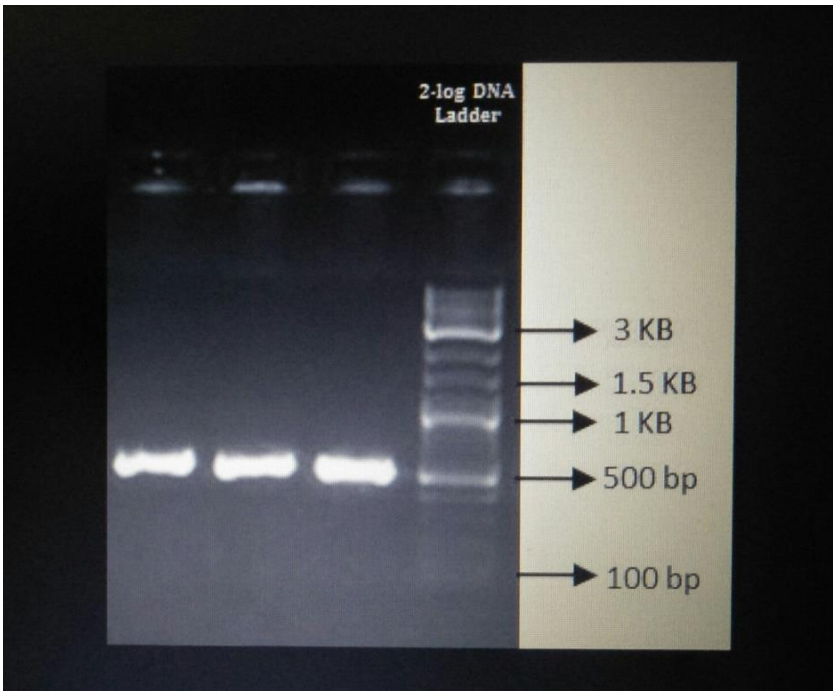
Nucleotide sequences captured from NCBI compared to the isolate MC 2 showed 99.8 per cent identity with 99 per cent query coverage to *M. anisopliae* isolate MaGX7002, *M. anisopliae* isolate MaGX02A02, *M. anisopliae* strain MA-58 and *M. anisopliae* isolate M161063. Accession number of MC 2 obtained from Bankit was MN538358.

4.1.3.2.2. ***Sequence Comparison of the Isolate MC 4***

Nucleotide databases of the sequences captured from NCBI when compared to the isolate MC 4 showed 99.79 per cent identity with 96 per cent query coverage to the sequences of *M. anisopliae* isolate BUM1900, *M. anisopliae* isolate strain M9, *M. anisopliae* isolate strain IIHR and *M. anisopliae* isolate M-63. Accession number of the isolate MC 4 acquired was MN538359.

4.1.3.2.3. ***Sequence Comparison of the Isolate MC 7***

Nucleotide sequence of the isolates MC 7 showed 100 per cent identity with 88 per cent query coverage to the sequences of *M. anisopliae* isolate BCC<THA>,



Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

Plate 4. Gel profile of *Metarhizium anisopliae* isolates

M. anisopliae strain TUMA02, *M. anisopliae* strain TUMA01 and *M. anisopliae* isolate FM-03. Accession number of the isolate MC 7 was MN538360.

4.1.3.2.4. Phylogenetic Analysis of Metarhizium anisopliae Isolates

A maximum livelihood phylogenetic tree was built by alignment of nucleotide sequences of three isolates of *M. anisopliae* (MC 2, MC 4 and MC 7) in the study and four sequences coding for ITS sequence of *Sterigmatomyces* sp. (A), *Aspergillus terreus* (B), *Talaromyces* sp. (C) and *Penicillium australicum* (D) available in the Genbank (Fig 1). The phylogenetic tree comprises of two clusters *ie*., A and MC 7 belongs to cluster 1 and rest others in cluster 2 which was further divided into two sub groups viz., sub group A and sub group B. Isolate MC 2 and MC 4 from Alappuzha district are assembled together with the isolates reported from other locations of India as well as from other countries. This grouping is supported by a bootstrap value of 100. Within a subgroup, these two isolates form a separate clusters and are closely related to each other. MC 2 and MC 4 as well as B and D are paralogs. MC 4 or A of cluster 1 and MC 4 or MC 2 or B or C or D of cluster 2 are orthologs.

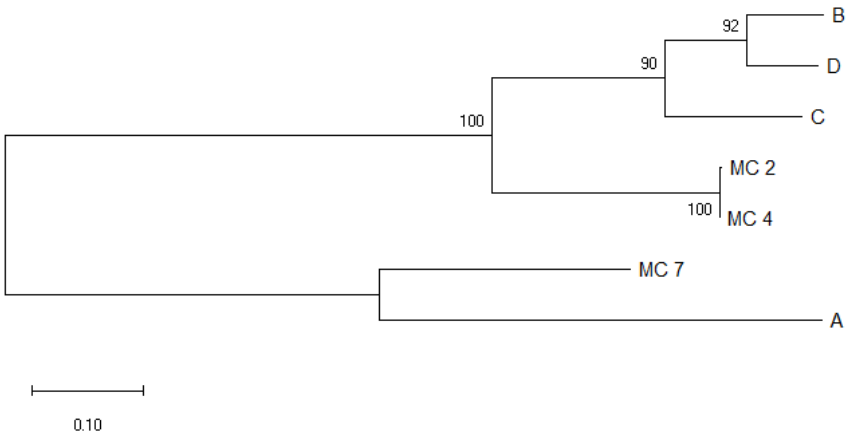


Fig 1. Phylogenetic analysis of *Metarhizium anisopliae* isolates

Table 6. Cultural and morphological characteristics of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Cultural characters	Colour of conidia	Shape of conidia	Size of conidia (µm)*	Arrangement of conidia	Size of phialides (µm)*
1.	MC 2	Sparse offwhite coloured mycelium with light olive green sporulation	Light olive green	Cylindrical dome shaped	3.53×1.61	Forming chains from tip of the phialide	6.61×1.51
2.	MC 4	Greenish mycelium with green sporulation	Green	Cylindrical dome shaped	3.72×1.38	Appears as chains as well as in clusters from tip of the phialide	4.72×1.32
3.	MC 7	Sparse white coloured mycelium with dark olive green sporulation	Dark olive green	Cylindrical dome shaped	4.05×1.70	Forms chains from tip of the phialide	7.01×1.50

* Mean of 25 observations

Table 7. Genomic sequences of *Metarhizium anisopliae* isolates

***M. anisopliae* isolates**	**Genomic sequences**
MC 2	ATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTCGAGGTCAACTA TAAAAAGTTGGGGGGTTTTACGGCAGTGGACCGCGCCGGGCTCCT GTTGCGAGTGCTTTACTACTGCGCAGAGGAGGGCCACGGCGAGA CCGCCAATTAATTTAAGGGACGGCTGTGCTGGAAAACCAGCCTCG CCGATCCCCAACACCAAGTCCACAGGGGACTTGAGGGGCGTAAT GACGCTCGAACAGGCATGCCCGCCAGAATACTGACGGGCGCAAT GTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACAT TACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAG AGATCCGTTGTTGAAAGTTTTGATTCATTTTTTTTAACCACTCAGA AGATACTTATTAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCG CGAAATCCCGCCGAAGCAACAATTAAAGGTATGATCACAGGGGT T
MC 4	ATTCGAGGTCACTATAAAAAGTTGGGGGGTTTTACGGCAGTGGAC CGCGCCGGGCTCCTGTTGCGAGTGCTTTACTACTGCGCAGAGGAG GGCCACGGCGAGACCGCCAATTAATTTAAGGGACGGCTGTGCTG GAAAACCAGCCTCGCCGATCCCCAACACCAAGTCCACAGGGGAC TTGAGGGGCGTAATGACGCTCGAACAGGCATGCCCGCCAGAATA CTGACGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAAT TCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGA TGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTTT TTAACCACTCAGAAGATACTTATTAAAAAATTCAGAAGGTTTGGG TCCCCGGCGGGCGCGAAGTCCCGCCGAAGCAACAATTAAAGGTA TGATCACAGGGGTTGGGAGTGGTTCTTCCGTTTTC
MC 7	GAGTTGGATAACTCGGTAATGATCCGGGCGCAGGGGAACGTGCT GTCCGGAAGATAAGGAGGATCCAACTCCCAACCCCTGTGAATCAT ACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACC CAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAA AAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAG TATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG TCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCA GCACAGCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTC CTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCGGCGCGGT CCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATC AGGTAGGACTACCCGCTGAACTT

Table 8. Sequence homology of *Metarhizium anisopliae* isolates in BLASTn analysis

***M. anisopliae* isolate**	**Description**	**Maximum score**	**Query coverage (%)**	**E value**	**Identity (%)**
MC 2	*M. anisopliae* isolate MaGX7002	904	99	0	99.80
	M. anisopliae isolate MaGX02A02	904	99	0	99.80
Accession number MN538358	*M. anisopliae* strain Ma-58	904	99	0	99.80
	M. anisopliae isolate M161063	902	99	0	99.80
MC 4	*M. anisopliae* isolate BUM1900	859	96	0	99.79
	M. anisopliae isolate strain M9	859	96	0	99.79
Accession number MN538359	*M. anisopliae* isolate strain IIHR	859	96	0	99.79
	M. anisopliae isolate M-63	859	96	0	99.79
MC 7	*M. anisopliae* isolate BCC<THA>	922	88	0	100
	M. anisopliae strain TUMA02	922	88	0	100
Accession number MN538360	*M. anisopliae* strain TUMA01	922	88	0	100
	M. anisopliae isolate FM-03	922	88	0	100

4.1.4. Selection of Suitable Cultural Medium for *Metarhizium anisopliae*

Screening for suitable medium for the growth of *Metarhizum anisopliae* revealed that PDA promoted better growth of isolates with mean radial growth ranging between 8.70 and 9 cm as against mean radial growth of 4.30 to 4.40 cm observed in SDA medium (Table 9). Based on the above results, PDA medium was selected for culturing of *M. anisopliae* isolates for further studies (Plate 5).

Table 9. Effect of cultural media on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Radial growth (cms)*	
		Sabauraud's dextrose agar medium (SDA)	Potato dextrose agar Medium (PDA)
1.	MC 2	4.40	8.80
2.	MC 4	4.40	9.00
3.	MC 7	4.30	8.70

4.2. *IN VITRO* SCREENING OF *Metarhizium anisopliae* ISOLATES FOR STRESS TOLERANCE

The three isolates of *M. anisopliae* were exposed to different levels of pH, salinity, temperature, drought, insecticides and fungicides for evaluating their tolerance to various abiotic stresses.

4.2.1. Screening of *Metarhizium anisopliae* Isolates for Acidity Tolerance

Effect of different pH conditions on the growth and sporulation of *M. anisopliae* is furnished in Tables 10 and 11. A significant difference in the growth was noticed among the isolates when exposed to lower pH levels (Plate 6).

At pH 6, highest mycelial weight of 5.63 g was recorded in the isolate MC 7. This was followed by MC 4 with mean mycelial weight of 5.56 g, both being on par with each other. MC 2 had the lowest mycelial weight of 5.46 g. When at pH of 5.5,

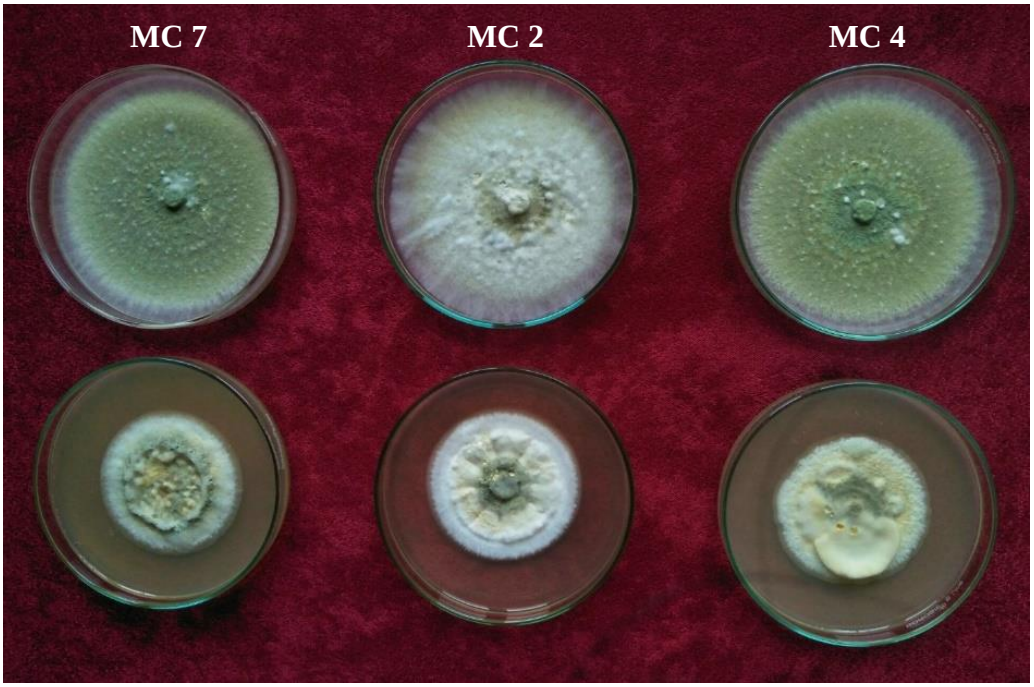


Plate 5. Selection of cultural medium for *Metarhizium anisopliae*

isolate MC 7 recorded a significant highest mycelial biomass of 4.78 g. It was followed by MC 2 (4.62 g) and MC 4 (4.60 g), both being at par. Similar results were also observed at pH value of 4.5, where the isolate MC 7 recorded the highest mycelial weight of 4.19 g compared to 3.53 and 3.79 g by MC 2 and MC 4 respectively. Isolate MC 7 once again recorded highest mean mycelial yield of 2.83 g while MC 2 and MC 4 registered mycelial weight of 2.27 and 2.56 at a lowest pH 3.5. At pH 3, the mycelial weights recorded by the isolates MC 2, MC 4 and MC 7 were 2.21, 2.33 and 2.54 g respectively. The highest mycelial biomass at pH 2.5 was observed in case of isolate MC 7 (1.15 g), followed by MC 2 (0.86 g) and MC 4 (0.10 g). The isolate MC 7 was significantly superior to remaining isolates in terms of mycelial weight at all pH levels evaluated except at pH 6.

Observations on the sporulation of *M. anisopliae* isolates at different pH levels revealed that all the isolates recorded high sporulation at pH levels 6 and 5.5. At a pH of 4.5, both MC 7 and MC 2 showed comparable sporulation whereas MC 4 showed medium sporulation. The isolate MC 7 showed high sporulation at pH 3.5 as well. In comparison, MC 2 showed medium sporulation whereas in case of MC 4, sporulation was sparse. At a lower pH of 3, isolate MC 7 recorded medium sporulation whereas the isolates MC 2 and MC 4 recorded sparse sporulation. None of the isolates produced spores at a lowest pH of 2.5.

4.2.2. Screening of *Metarhizium anisopliae* Isolates for Salinity Tolerance

Salinity tolerance of the three *M. anisopliae* isolates was studied by evaluating the per cent growth inhibition of the isolates at different concentrations of NaCl (Table 12).

At 0.5 M concentration of NaCl in the cultural medium, the isolates differed significantly, with growth inhibition ranged from 6.33 to 54.71 per cent. The isolate MC 2 showed the lowest growth inhibition of 6.33 per cent which was significantly superior to the growth inhibition observed in MC 4 (50.32 %) and MC 7 (54.71 %).

The isolate, MC 2 consistently registered the lowest growth inhibition at higher salt concentrations of 1 M, 1.5 M and 2 M, with values of 50.72, 58.84 and 94.10 per cent respectively. At the same salt concentrations, growth inhibition was in the order 58.54, 89.21 and 100.00 per cent for MC 4 and 60.71, 89.60 and 100.00 per cent for MC 7 respectively (Plate 7).

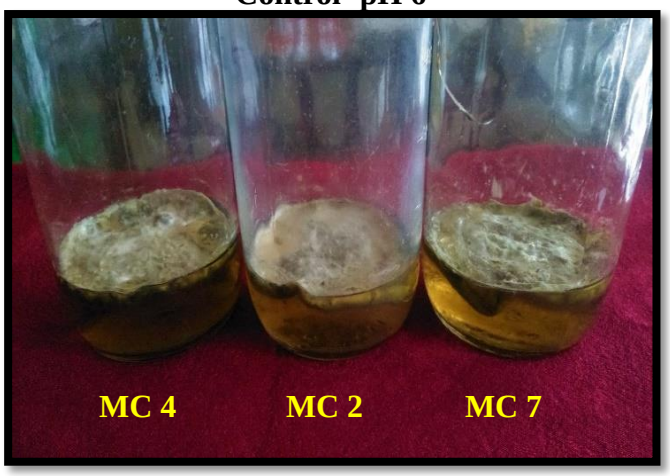
Sporulation of the isolates with respect to the salt concentrations was also observed (Table 13). At the lowest salt concentration of 0.5 M, the isolate MC 2 showed medium sporulation while the isolate MC 4 exhibited sparse sporulation and no sporulation was observed in case of MC 7. When the concentration of NaCl in the medium was 1 M, isolate MC 2 gave sparse sporulation whereas only mycellial growth was observed in the other two isolates. None of the isolates showed sporulation at higher salt concentrations (1.5 and 2 M).

4.2.3. Screening of *Metarhizium anisopliae* Isolates for Temperature Tolerance

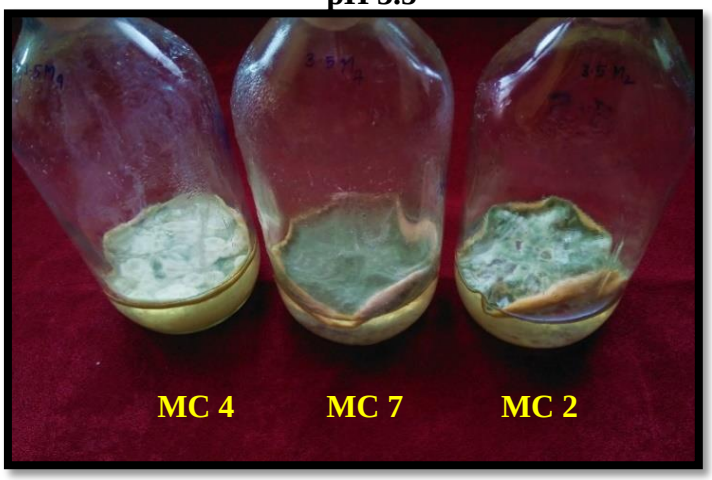
All the three isolates of *M. anisopliae* were subjected to five different levels of temperatures in order to identify the best isolate that can withstand high temperatures. The results are furnished in Tables 14 and 15.

At 25^0C, the highest mycellial biomass of 4.01g was produced by the isolate MC 7 followed by MC 2 with 3.83 g mycelial weight. MC 4 yielded the lowest mycelial weight of 3.72 g at 25^0C. At a temperature of 27^0 C, all the isolates showed an increase in biomass, which ranged from 5.46 to 5.63 g. The isolates MC 7 and MC 4 recorded highest mycellial biomass of 5.63 g and 5.56 g respectively and the lowest biomass was recorded in MC 2 (5.46 g). MC 7 consistently registered highest mycellial mass of 2.79 g at 30^0 C followed by MC 4 (2.48 g) and MC 2 (2.34 g). At highest temperature of 37^0C, the isolate MC 7 recorded highest biomass production of 1.28 g and followed by MC 4 with a mycellial weight of 0.41 g. Lowest mycellial weight of 0.38 g was observed for the isolate, MC 2. None of the isolates could survive at 40^0C (Plate 8).

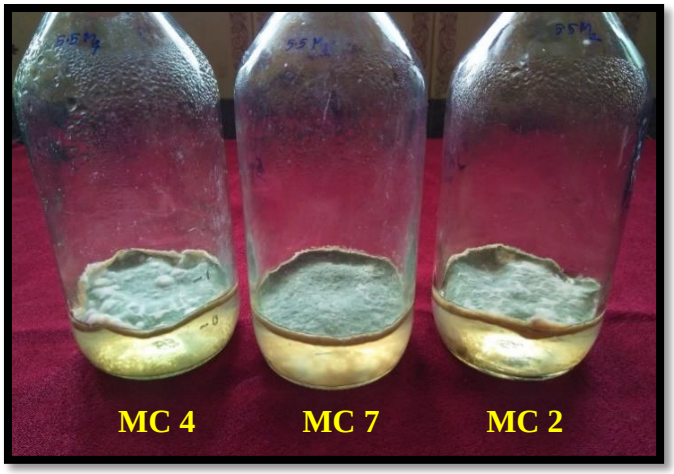
Control- pH 6



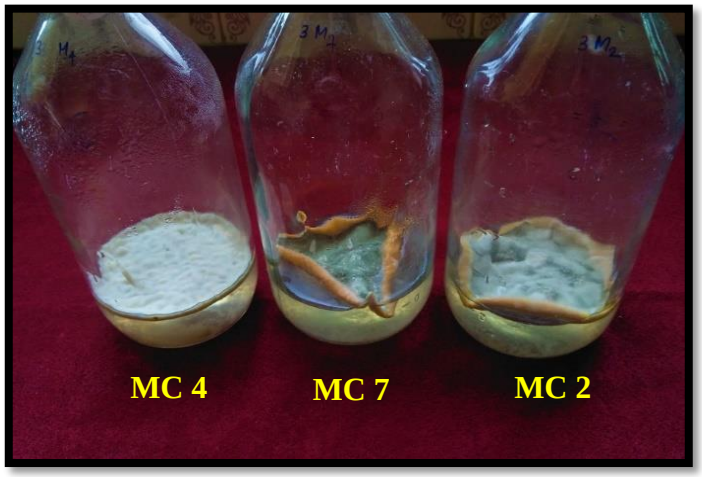
pH 3.5



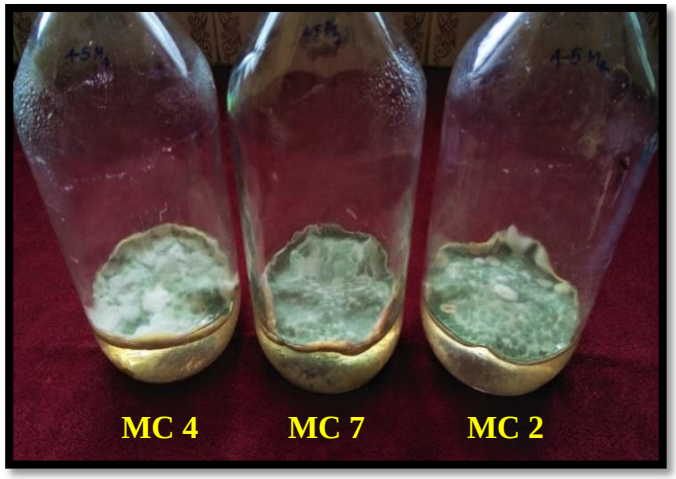
pH 5.5



pH 3



pH 4.5



pH 2.5

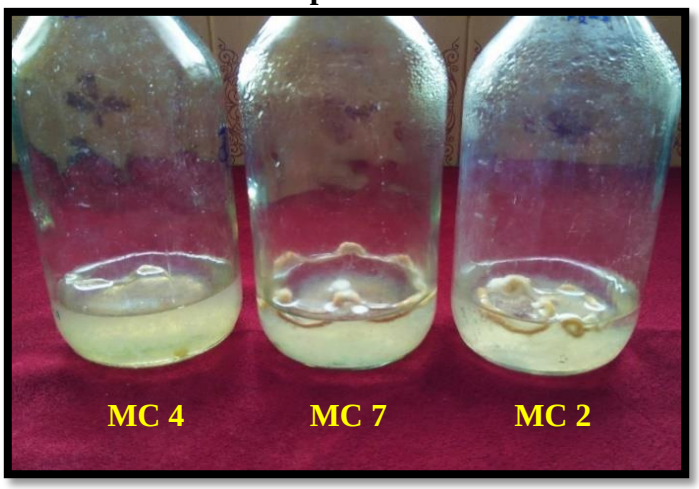


Plate 6. Growth and sporulation of *Metarhizium anisopliae* isolates at different pH levels

Table 10. Effect of pH on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Mycelial weight (g/100 ml) *					
		pH 6	pH 5.5	pH 4.5	pH 3.5	pH 3	pH 2.5
1.	MC 2	5.46[b]	4.62[b]	3.53[c]	2.27[c]	2.21[c]	0.86[b]
2.	MC 4	5.56[a]	4.60[b]	3.79[b]	2.56[b]	2.33[b]	0.10[c]
3.	MC 7	5.63[a]	4.78[a]	4.19[a]	2.83[a]	2.54[a]	1.15[a]
CD (0.05)		0.074	0.109	0.121	0.122	0.116	0.103

* Mean of five replications

Table 11. Effect of pH on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation at different pH levels					
		6	5.5	4.5	3.5	3	2.5
1.	MC 2	+++	+++	+++	++	+	-
2.	MC 4	+++	+++	++	+	+	-
3.	MC 7	+++	+++	+++	+++	++	-

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

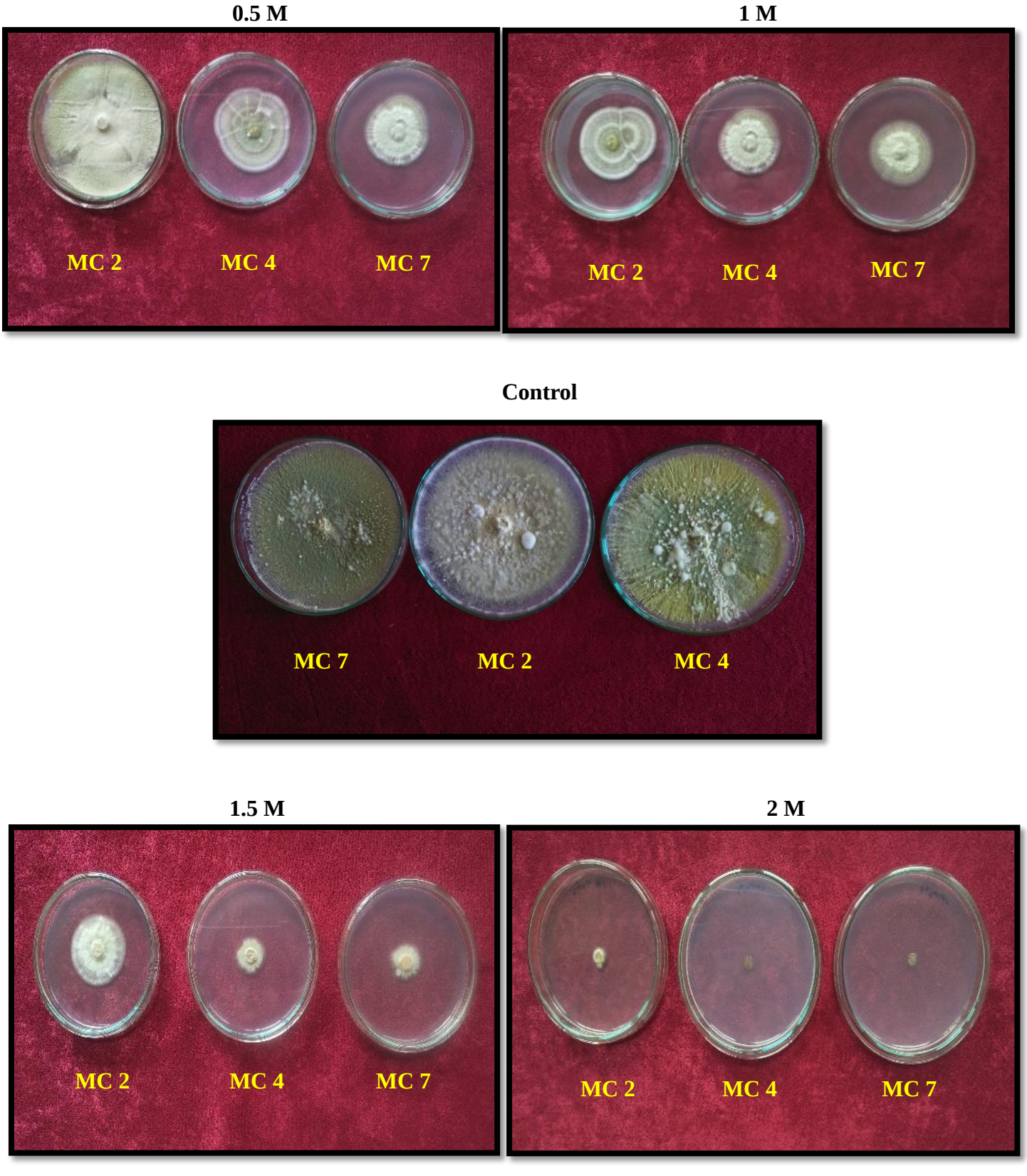


Plate 7. Effect of Nacl on the growth and sporulation of *Metarhizium anisopliae* isolates

Table 12. Effect of salinity on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *			
		0.50 M	**1.00 M**	**1.50 M**	**2.00 M**
1.	MC 2	6.33(3.57)^{c}	50.72(30.50)^{c}	58.84(36.06)^{b}	94.10(70.16)^{b}
2.	MC 4	50.32(30.25)^{b}	58.50(35.77)^{b}	89.21(63.16)^{a}	100.00(90.04)^{a}
3.	MC 7	54.71(33.22)^{a}	60.71((37.41)^{a}	89.60(63.67)^{a}	100.00(90.04)^{a}
CD (0.05)		0.816	0.884	1.726	1.217

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 13. Effect of salinity on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different concentrations of NaCl				
		Control	**0.5 M**	**1 M**	**1.5 M**	**2 M**
1.	MC 2	+++	++	+	-	-
2.	MC 4	+++	+	-	-	-
3.	MC 7	+++	-	-	-	-

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

At all temperatures, the isolate MC 7 recorded significantly superior biomass except at 27^0C where it was on par with MC 4. Isolate MC 4 with mean mycelial weight ranging from 0.41 to 3.72 g/100 ml was significantly superior to MC 2, which recorded lowest mycelial mass at all temperatures evaluated except at 25^0C.

Sporulation of all the isolates was also observed at different temperatures. At 25^0C, none of the isolates recorded sporulation. However all the isolates recorded high sporulation at 27^0C. At 30°C, all the isolates had medium sporulation. Isolate MC 7 alone showed high sporulation even at 35ºC while the remaining two isolates had sparse sporulation. At 37ºC, isolate MC 7 recorded sparse sporulation while no sporulation was observed in both MC 4 and MC 2.

4.2.4. Screening of *Metarhizium anisopliae* Isolates for Drought Tolerance

Results of the experiment conducted to screen the drought tolerant isolate are depicted in the Tables 16 and 17. When the PDA medium was amended with 10 per cent PEG concentration, the isolates MC 7 and MC 2 recorded highest mycellial weights of 2.40 g and 2.39 g respectively and were on par with each other. At the same concentration of PEG, isolate MC 4 recorded lowest mycellial mass of 2.12 g. When the isolates were subjected to 20 per cent PEG concentration, peak mycellial mass of 1.83 g was registered by the isolate MC 2 and was followed MC 7 with a mycelial mass of 1.54 g. Lowest mycelial mass of 1.41 g was recorded by the isolate MC 4. Similar trend was also observed at 30 per cent PEG concentration, where isolate MC 2 continued to record highest growth (1.38 g). At this concentration, both MC 7 and MC 4 registered significantly lower biomass of 1.16 and 0.91 g respectively than MC 2. When the media were amended with highest concentration of PEG (35 per cent), highest mycelial mass of 0.93 g was observed in case of MC 2, followed by MC 7 with 0.37 g mycellial weight. The isolate MC 4 couldn't survive at the same PEG concentration (Plate 9).

Sporulation of the isolates at different levels of PEG was also recorded. The

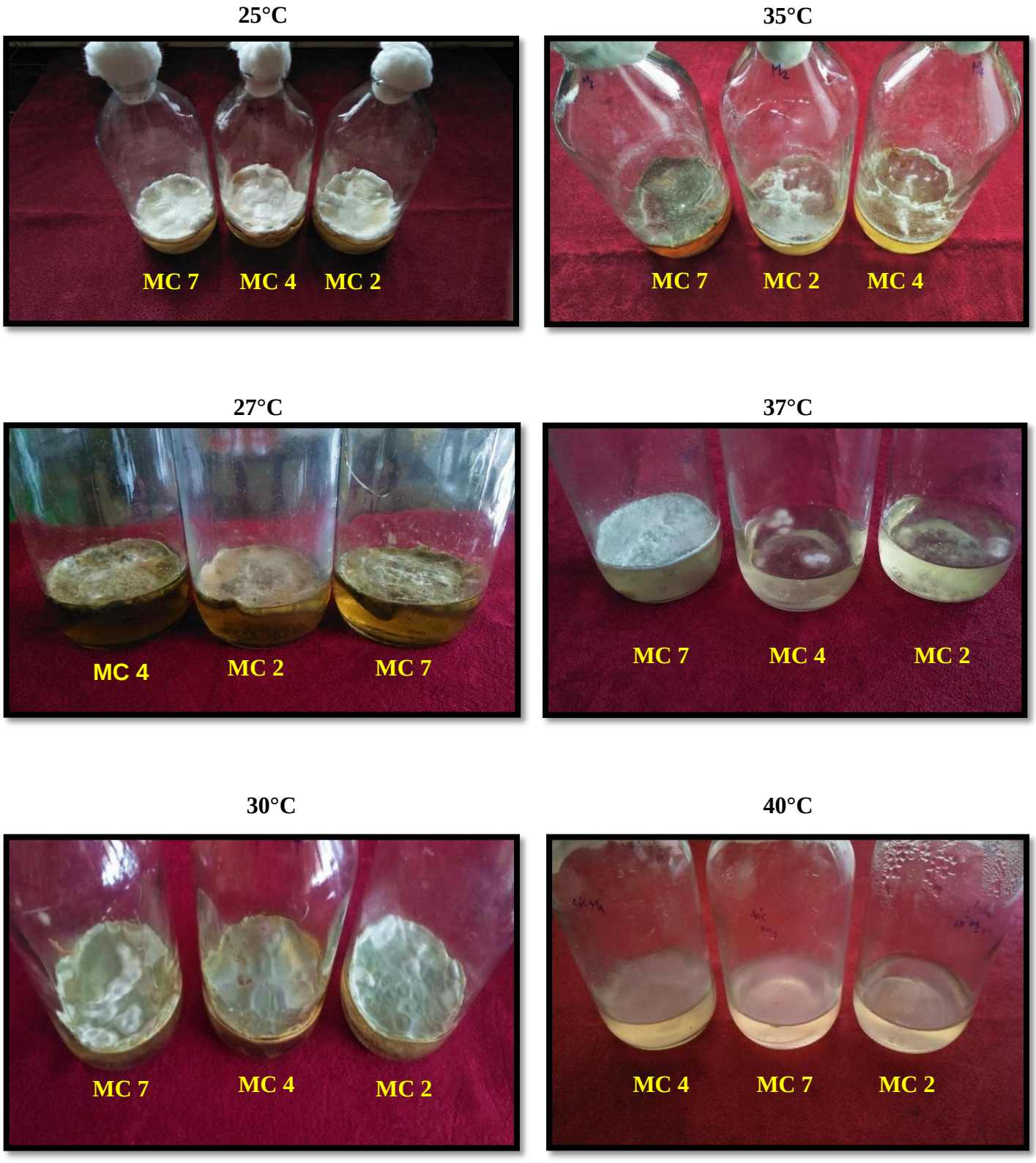


Plate 8. Growth and sporulation of *Metarhizium anisopliae* at different temperatures

Table 14. Effect of temperature on the growth of *Metarhzium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Mycelial weight (g/100 ml) *					
		25°C	**27°C**	**30°C**	**35°C**	**37°C**	**40°C**
1.	MC 2	3.83[b]	5.46[b]	2.34[b]	1.24[c]	0.38[c]	0.00
2.	MC 4	3.72[c]	5.56[a]	2.48[b]	1.80[b]	0.41[b]	0.00
3.	MC 7	4.01[a]	5.63[a]	2.79[a]	2.42[a]	1.28[a]	0.00
CD (0.05)		0.081	0.074	0.155	0.108	0.046	-

* Mean of five replications

Table 15. Effect of temperature on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different temperatures					
		25°C	**27°C**	**30°C**	**35°C**	**37°C**	**40°C**
1.	MC 2	-	+++	++	+	-	-
2.	MC 4	-	+++	++	+	-	-
3.	MC 7	-	+++	++	+++	+	-

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

isolate MC 7 showed high sporulation at 10 per cent PEG concentration whereas MC 2 had moderate sporulation. No sporulation was observed in case of MC 4. At 20 per cent PEG concentration, isolates MC 2 and MC 7 exhibited medium sporulation and MC 4 had sparse sporulation. Isolate MC 2 alone exhibited sparse sporulation at PEG concentration of 30 per cent. No sporulation was noticed in any of the three isolates at 35 per cent concentration of PEG.

Among the three isolates tested, MC 2 recorded significantly highest mycelial biomass at higher concentration of PEG and was considered for further studies.

4.2.5. Screening of *Metarhizium anisopliae* Isolates for Insecticides and Fungicides Tolerance

An experiment was conducted to assess the tolerance of *M. anisopliae* isolates to selected insecticides and fungicides through poison food technique and the results are presented in the Tables 18 to 29.

4.2.5.1. Screening of Isolates for Compatibility with Spinosad

Isolates of *M. anisopliae* were allowed to grow in PDA medium amended with different doses of spinosad *ie*., 0.28, 0.33 and 0.38 g/L, to evaluate their compatibility with spinosad. Percent inhibition of the isolates at different doses of spinosad are given in the Table 18.

At the lowest dose of spinosad (0.28 ml/L), the isolate MC 7 recorded lowest growth inhibition (3.62 %). This was followed by MC 4 and MC 2 with 4.42 and 4.61 per cent growth inhibition respectively. At recommended dose of spinosad (0.33 ml/L), least growth inhibition was observed in case of the isolate MC 7 (8.11 %) followed by MC 4 and MC 2 (10.71 and 16.64 % respectively). Similarly, at the highest dose of spinosad (0.38 ml/L), the isolate MC 7 reported the lowest growth inhibition of 11.41 per cent which was significantly lower to the growth inhibitions of both MC 2 and MC 4 (17.40 and 19.23 % respectively) [Plate 10].

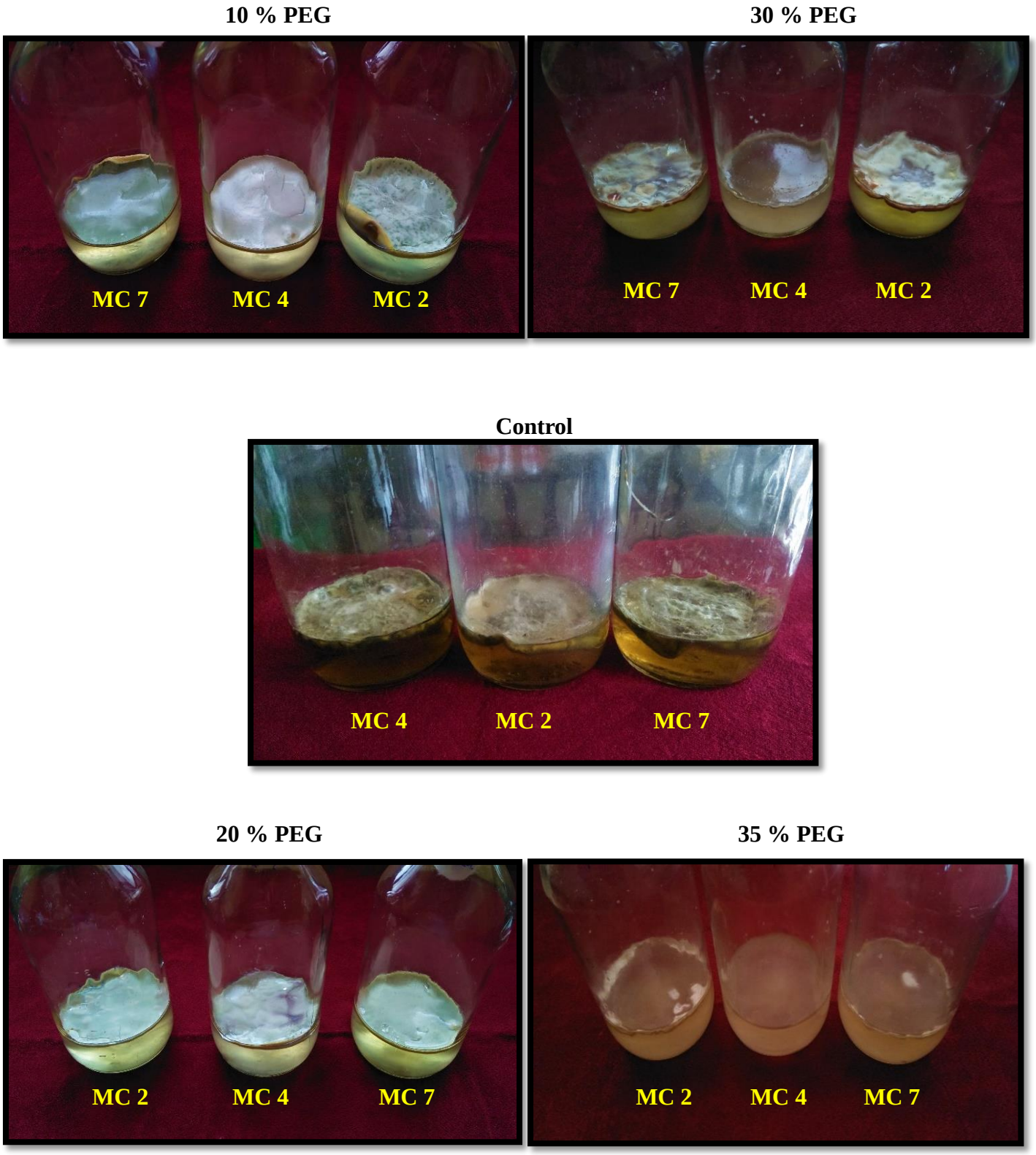


Plate 9. Effect of PEG on the growth and sporulation of *Metarhizium anisopliae* isolates

Table 16. Effect of drought on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Mycelial weight (g/100 ml) *			
		10 % PEG	20 % PEG	30 % PEG	35 % PEG
1.	MC 2	2.39[a]	1.83[a]	1.38[a]	0.93[a]
2.	MC 4	2.12[b]	1.41[c]	0.91[c]	0.00[c]
3.	MC 7	2.40[a]	1.54[b]	1.16[b]	0.37[b]
CD (0.05)		0.074	0.128	0.195	0.177

* Mean of five replications

Table 17. Effect of drought on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different concentrations of PEG				
		Control	10 %	20 %	30 %	35 %
1.	MC 2	+++	++	++	+	-
2.	MC 4	+++	-	+	-	-
3.	MC 7	+++	+++	++	-	-

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

Effect of spinosad on the sporulation of isolates was also recorded and is presented in the Table 19. At the lowest dose of 0.28 ml/L, spinosad had little effect on sporulation of the isolates. However, at recommended dose of 0.33 ml/L, isolates MC 2 and MC 7 exhibited medium sporulation while isolate MC 4 had sparse sporulation. When the isolates were grown in PDA amended with highest dose of spinosad (0.38 ml/L), the isolate MC 7 recorded medium sporulation while no sporulation was observed in case of the isolates MC 2 and MC 4.

4.2.5.2. Screening of Isolates for Compatibility with Cypermethrin

Isolates of *M. anisopliae* were screened for compatibility with cypermethrin at different doses of 0.35, 0.40 and 0.45 ml/L. The isolates exhibited a considerable growth inhibition at different doses of cypermethrin (Table 20). When PDA medium was amended with the lowest dose of 0.35 ml/L, isolate MC 4 recorded least growth inhibition (51.51 %) whereas the isolates MC 2 and MC 7 recorded highest growth inhibition (53 and 53.74 %) and were on par with each other. At recommended dose of cypermethrin (0.40 ml/L), isolate MC 2 registered the lowest growth inhibition of 56.23 per cent followed by MC 7 and MC 4 (59.20 and 61.90 %). MC 2 had recorded significantly lower inhibition than both MC 7 and MC 4, being significantly different from each other. All the isolates recorded more than 60 per cent growth inhibition at 0.45 ml/L dose. Isolates MC 7 and MC 4 recorded higher growth inhibitions (62.92 and 64.04 per cent respectively) and were on par with other (Plate 10).

Cypermethrin had a negative impact on the sporulation by the three isolates (Table 21). Isolates MC 4 and MC 7 showed only medium sporulation even at the lowest dose of 0.35 ml/L while MC 2 recorded sparse sporulation. Both MC 4 and MC 7 recorded sparse sporulation at the dose of 0.40 ml/L and no sporulation was observed in case of MC 2 at the same dose. At 0.45 ml/L dose, the isolate MC 7 showed medium sporulation whereas in case of MC 2 and MC 4, no sporulation was noticed.

Table 18. Effect of spinosad on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.28 ml/L	**0.33 ml/L**	**0.38 ml/L**
1.	MC 2	4.61(2.97)^{a}	16.64(9.59)^{a}	17.40(10.02)^{b}
2.	MC 4	4.42(2.54)^{ab}	10.71(6.16)^{b}	19.23(11.10)^{a}
3.	MC 7	3.62(2.12)^{b}	8.11(4.67)^{c}	11.41(6.59)^{c}
CD (0.05)		0.600	0.599	0.746

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 19. Effect of spinosad on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different concentrations of spinosad			
		Control	**0.28 ml/L**	**0.33 ml/L**	**0.38 ml/L**
1.	MC 2	+++	+++	++	-
2.	MC 4	+++	+++	+	-
3.	MC 7	+++	+++	++	++

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

4.2.5.3. Screening of Isolates for Compatibility with Imidacloprid

Screening of *Metarhizium anisopliae* isolates was carried out to assess their compatibility with the imidacloprid at different doses of 0.05, 0.10 and 0.15 g/L (Table 22). Significantly lowest growth inhibition of 7.10 per cent was recorded in case of MC 7 at 0.05 g/L, followed by MC 4 (10. 30 %) and highest growth inhibition was observed in the isolate MC 2 (13.64 %). At 0.10 g/L dose, isolate MC 7 was the least inhibited at 11.93 per cent followed by MC 4 with 16.70 per cent and MC 2 with 19.20 per cent growth inhibition. When the dose was increased to 0.15 g/L, highest growth was observed in the isolate MC 7 with least inhibition (14.44 %). It was followed by isolates MC 4 and MC 2 with 17.43 and 21.50 per cent growth inhibition respectively. MC 7 consistently had significantly lower per cent growth inhibition at all doses of imidacloprid, which was followed by MC 4. The isolate MC 2 recorded significantly higher degree of growth inhibition than both MC 7 and MC 4 at all doses of imidacloprid (Plate 11).

Effect of imidacloprid on the sporulation of isolates at different doses was also noticed and presented in the Table 23. Sporulation of isolate MC 7 was increased with the increase in dose of imidacloprid from 0.05 to 0.15 g/L. But in case of MC 2 and MC 4, sporulation showed a reducing trend with increase in dose of imidacloprid. At the dose of 0.05 g/L, isolates MC 2 and MC 4 produced sparse sporulation whereas isolate MC 7 had medium sporulation. Similarly at 0.10 g/l, the isolate MC 7 showed high sporulation followed by MC 4 with sparse sporulation. No sporulation was observed in the isolate MC 2 at the same dose. At the highest dose of 0.15 g/L imidacloprid, there was no sporulation noticed in the isolates MC 2 and MC 4 but high sporulation was observed in the case of MC 7.

4.2.5.4. Screening of Isolates for Compatibility with Chlorantraniliprole

Three isolates of *M. anisopliae* were screened to evaluate compatibility with different doses of chlorantraniliprole *ie*., 0.25, 0.30 and 0.35 ml/L. The results are

Plate 10. Effect of spinosad and cypermethrin on the growth and sporulation of *Metarhizium anisopliae* isolates

Table 20. Effect of cypermethrin on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.35 ml/L	0.40 ml/L	0.45 ml/L
1.	MC 2	53.00(32.22)[a]	56.23(33.72)[c]	60.71(36.90)[b]
2.	MC 4	51.51(30.75)[b]	61.90(38.48)[a]	64.04(40.11)[a]
3.	MC 7	53.74(33.00)[a]	59.20(36.03)[b]	62.92(38.40)[a]
CD (0.05)		1.269	1.308	1.269

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 21. Effect of cypermethrin on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different concentrations of cypermethrin			
		Control	0.35 ml/L	0.40 ml/L	0.45 ml/L
1.	MC 2	+++	+	-	-
2.	MC 4	+++	++	+	-
3.	MC 7	+++	++	+	++

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

are presented in the Table 24.

At the lowest dose of (0.25 ml/L), chlorantraniliprole had no inhibition effect on both MC 2 and MC 7. At the same dosage, an inhibition of 1.85 per cent was observed in case of the isolate MC 4. Similarly, at the recommended dose of chlorantraniliprole (0.30 ml/L), growth of isolate MC 7 remained unaffected by the insecticide but growth of isolates MC 2 and MC 4 were inhibited to the extent of 2.96 and 4.81 per cent respectively. All the three isolates differed from each other significantly. At the highest dose of 0.35 ml/L, isolate MC 7 recorded highest growth with least inhibition (1.11 %) followed by MC 4 and MC 2 (7.77 and 8.88 %) which were on par with each other (Plate 11).

The sporulation of the isolates also differed with respect to different doses of chlorantraniliprole (Table 25). All the isolates showed high sporulation at 0.25 ml/L dose. At the dose of 0.30 ml/L, isolate MC 2 exhibited moderate sporulation while the other two isolates maintained high sporulation. Addition of chlorantraniliprole at 0.35 ml/L to PDA medium, resulted in medium sporulation in MC 7 and sparse sporulation in both MC 2 and MC 4.

4.2.5.5. Screening of Isolates for Compatibility with Copper Oxychloride

Compatibility of isolates with the copper oxychloride was assessed at three different doses of 0.20, 0.25 and 0.30 g/L (Table 26). At the lowest dose of 0.20 g/L, copper oxychloride caused lowest growth inhibition of 1.11 per cent in the isolate MC 2 while isolates MC 4 and MC 7 recorded growth inhibition to the tune of 4.81 and 2.96 respectively and these isolates differed significantly from each other.

Isolate MC 2 once again recorded the lowest value of 2.59 per cent when exposed to dose at 0.25 g/L. This was significantly lower than that of both MC 4(5.92 %) and MC 7 (4.07 %). A similar trend was discernible at the highest dose of 0.30 g/L as well, with the isolate MC 2 registering the lowest growth inhibition of 3.70 per cent. This was followed by the isolates MC 7 and MC 4 with 5.18 and 7.03 per cent

Table 22. Effect of imidacloprid on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.05 g/L	**0.10 g/L**	**0.15 g/L**
1.	MC 2	13.64(7.87)[a]	19.20(11.10)[a]	21.50(12.41)[a]
2.	MC 4	10.30(5.95)[b]	16.70(9.59)[b]	17.43(10.02)[b]
3.	MC 7	7.10(4.03)[c]	11.93(6.80)[c]	14.44(8.30)[c]
CD (0.05)		0.738	0.610	0.611

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 23. Effect of imidacloprid on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different concentrations of imidacloprid			
		Control	**0.05 g/L**	**0.10 g/L**	**0.15 g/L**
1.	MC 2	+++	+	-	-
2.	MC 4	+++	+	+	-
3.	MC 7	+++	++	+++	+++

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

growth inhibition respectively. All the three isolates differed significantly from each other at all doses of copper oxychloride (Plate 12).

Variation in the sporulation of isolates at different doses of copper oxychloride was also noticed (Table 27). At the lowest dose of 0.20 g/L, the isolates MC 4 and MC 7 exhibited high sporulation while no sporulation was observed for the isolate MC 2. However MC 2 along with MC 4 showed medium sporulation at dose of 0.25 g/L whereas, high sporulation was observed in case of isolate MC 7. At highest dose of 0.30 g/L, isolate MC 2 and MC 4 were observed with sparse sporulation while isolate MC 7 had medium sporulation.

4.2.5.6. Screening of Isolates for Compatibility with Carbendazim

Metarhizium anisopliae isolates were screened for assessing their compatibility with carbendazim at different doses of 0.50, 1 and 1.50 g/L (Table 28).

Isolate MC 7 recorded highest radial growth in the PDA medium amended with carbendazim at the rate of 0.50 g/L, exhibiting 58.51 per cent inhibition. The isolate MC 2 recorded growth inhibition of 86.33 per cent and isolate MC 4 had the highest growth inhibition of 93.40 per cent. The three isolates differed from each other in terms of inhibition of growth by carbendazim. Complete inhibition was observed in all isolates at higher doses of 1 and 1.50 g/L (Plate 12).

Sporulation of isolates was also affected by carbendazim (Table 29). Isolate MC 7 showed high sporulation at 0.50 g/L dosage whereas isolate MC 2 had medium sporulation and no sporulation was noticed in the isolate MC 4. At higher doses (1 and 1.5 g/L), none of the isolates exhibited sporulation.

4.2.5.7. Screening of Isolates for Compatibility with Hexaconazole

The fungicide hexaconazole was also evaluated against *M. anisopliae* isolates at different doses of 1.50, 2 and 2.50 ml/L for compatibility. Growth of all isolates was completely inhibited by hexaconazole at all doses tried.

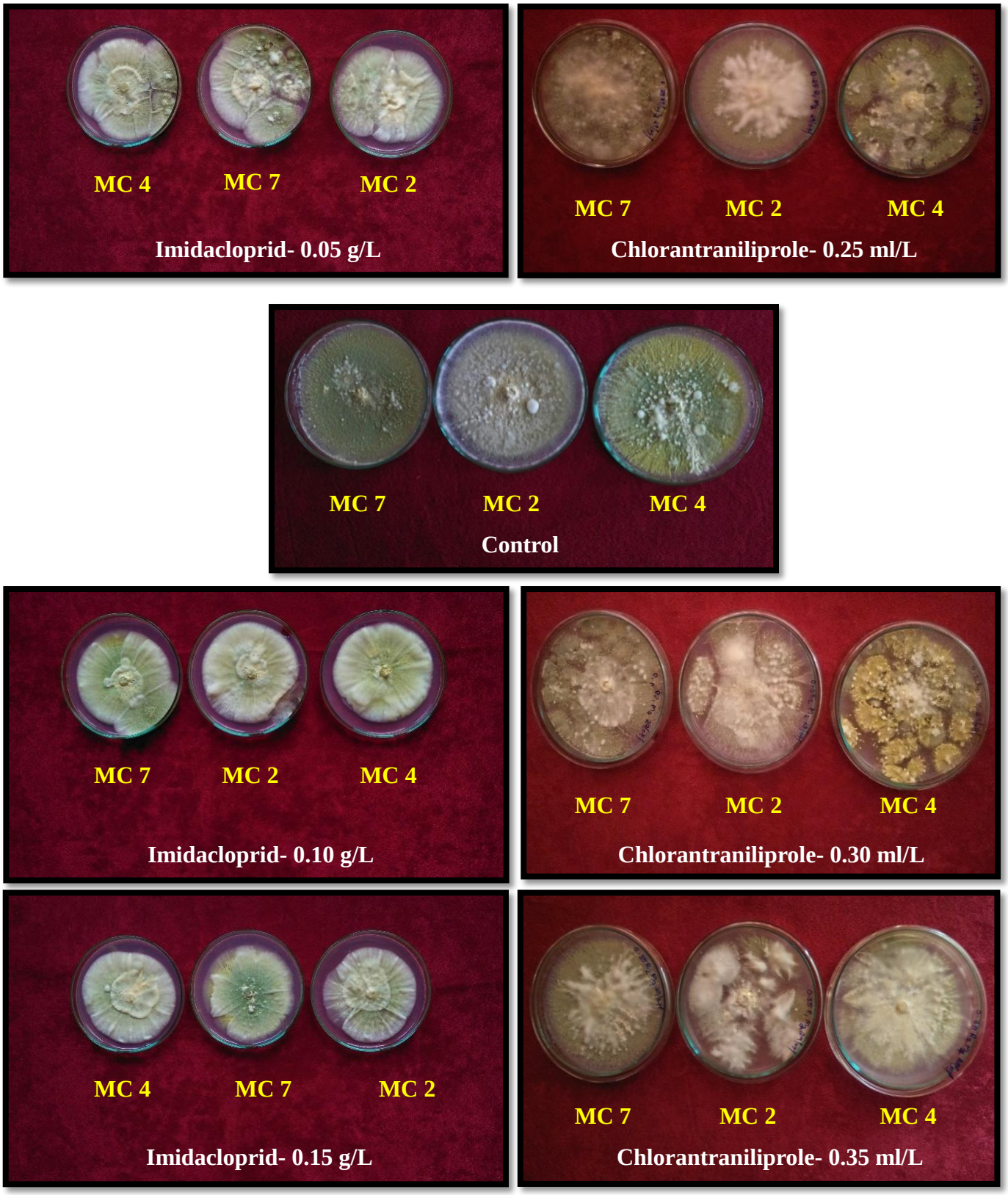


Plate 11. Effect of imidacloprid and chlorantraniliprole on the growth and sporulation of *Metarhizium anisopliae* isolates

Table 24. Effect of chlorantraniliprole on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.25 ml/L	0.30 ml/L	0.35 ml/L
1.	MC 2	0.00(0.00)^{b}	2.96(1.69)^{b}	8.88(4.88)^{a}
2.	MC 4	1.85(1.06)^{a}	4.81(2.75)^{a}	7.77(4.45)^{a}
3.	MC 7	0.00(0.00)^{b}	0.00(0.00)^{c}	1.11(0.84)^{b}
CD (0.05)		0.424	0.600	0.600

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 25. Effect of chlorantraniliprole on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different doses of chlorantraniliprole			
		Control	0.25 ml/L	0.30 ml/L	0.35 ml/L
1.	MC 2	+++	+++	++	+
2.	MC 4	+++	+++	+++	+
3.	MC 7	+++	+++	+++	++

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

4.3. BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae*

The two isolates of *M. anisopliae* namely, MC 7 and MC 2 were identified as most tolerant to temperature as well as drought respectively based on the results of previous experiments. Hence these isolates were subjected to biochemical analysis in order to assess the activity of various enzymes like esterase, catalase, peroxidase and a biomolecule trehalose. The variation in biochemical profile of the isolate MC 7 under different temperature regimes as well as that of MC 2 under varied moisture regimes were analyzed and are presented in Tables 30 and 31.

4.3.1. Total Protein Content in the Stress Tolerant Isolates of *Metarhizium anisopliae*

The total protein content of thermotolerant isolate MC 7 at three different temperatures was estimated (Table 30). Protein content was increased at higher levels of temperature. The isolate MC 7 recorded protein content of 4.410 mg/ml at 27°C which increased to 4.600 mg/ml at 35°C and again to 5.721 mg/ml at 37°C. But there was no significant difference in total protein content at both 27 and 35ºC. At the highest temperature of 37ºC, the protein content was significantly higher to those at lower temperatures.

Total protein content estimated in drought tolerant isolate MC 2 decreased from 4.27 mg/ml at 20 per cent concentration of PEG to a significantly lower protein content of 2.831 mg/ml at 35 per cent of PEG (Table 31). Control (without PEG) recorded highest quantity of protein with a value of 5.02 mg/ml, which was significantly higher to the values obtained at higher concentration of PEG.

4.3.2. Activity of Intracellular Enzyme Esterase in the Stress Tolerant Isolates

Mycelia of thermotolerant isolate grown at different temperatures of 27 (control), 35 and 37ºC and drought tolerant isolate at different concentrations of PEG such as 20 and 35 per cent and control (without PEG) were assessed in order to

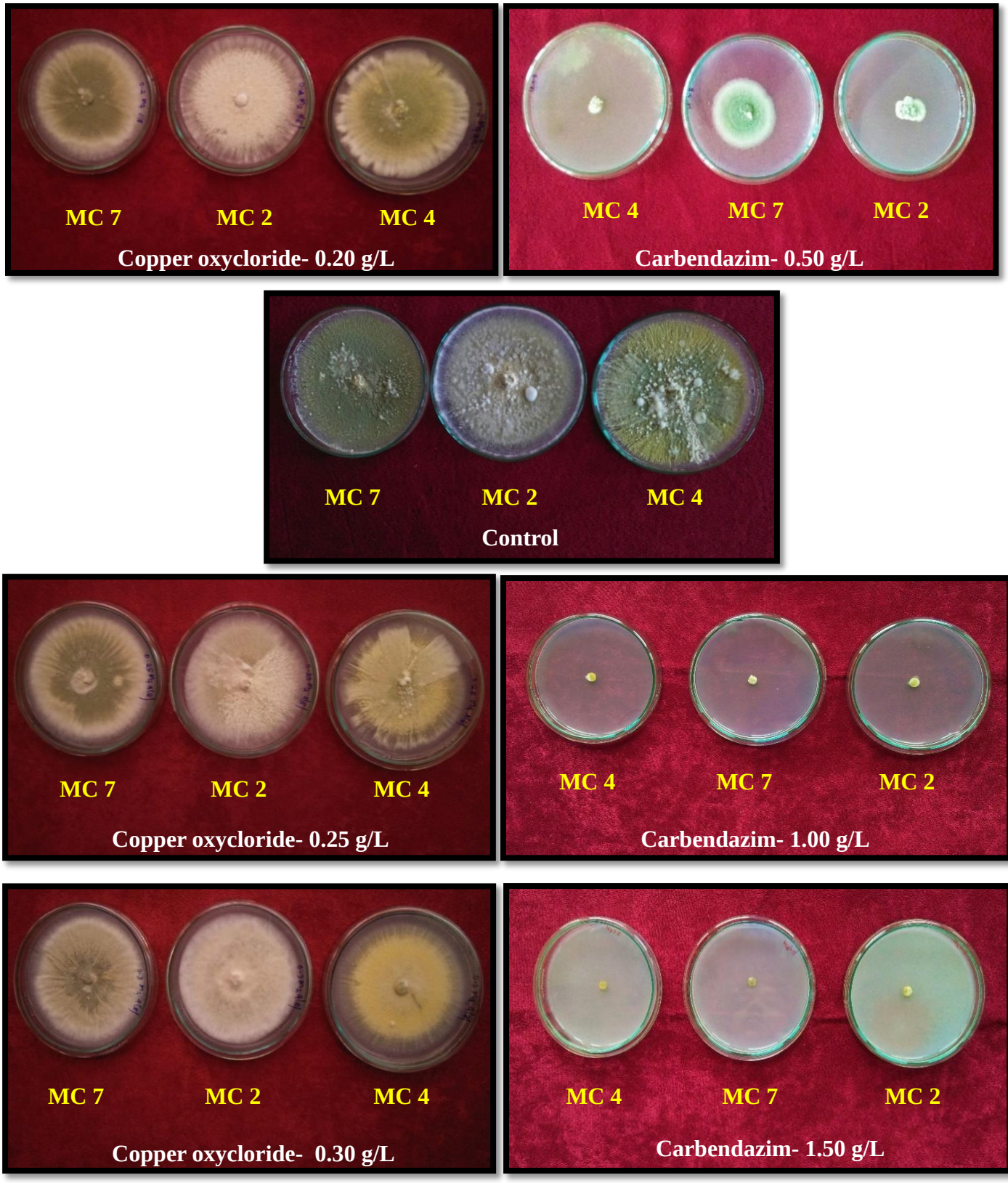


Plate 12. Effect of copper oxychloride and carbendazim on the growth and sporulation of *Metarhizium anisopliae* isolates

Table 26. Effect of copper oxychloride on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.20 g/L	**0.25 g/L**	**0.30 g/L**
1.	MC 2	1.11 (0.63)^{c}	2.59(1.48)^{c}	3.70(2.12)^{c}
2.	MC 4	4.81(1.69)^{b}	5.92(3.39)^{a}	7.03(4.03)^{a}
3.	MC 7	2.96(2.75)^{a}	4.07(2.33)^{b}	5.18(2.97)^{b}
CD (0.05)		0.600	0.735	0.735

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 27. Effect of copper oxychloride on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different doses of copper oxychloride			
		Control	**0.20 g/L**	**0.25 g/L**	**0.30 g/L**
1.	MC 2	+++	-	++	+
2.	MC 4	+++	+++	++	+
3.	MC 7	+++	+++	+++	++

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

Table 28. Effect of carbendazim on the growth of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Percent inhibition over control *		
		0.50 g/L	1.00 g/L	1.50 g/L
1.	MC 2	86.33 (59.61)[b]	100.00	100.00
2.	MC 4	93.40 (68.38)[a]	100.00	100.00
3.	MC 7	58.51 (35.77)[c]	100.00	100.00
CD (0.05)		1.487	-	-

* Mean of five replications

Values given in the parentheses are arcsine transformed values

Table 29. Effect of carbendazim on the sporulation of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Sporulation of isolates at different doses of carbendazim			
		Control	0.50 g/L	1.00 g/L	1.50 g/L
1.	MC 2	+++	++	-	-
2.	MC 4	+++	-	-	-
3.	MC 7	+++	+++	-	-

+++ : high sporulation, ++ **:** medium sporulation, + **:** sparse sporulation, - **:** no sporulation

to estimate variation in the activity of an intracellular enzyme esterase (Table 30). There was a significant difference found in the esterase activity of isolate MC 7 at different temperatures of 27, 35 and 37ºC within a range of 0.012 to 0.036 µmol/min/mg protein. The isolate MC 7 at optimum temperature of 27°C (control) had significantly highest esterase activity (0.036 µmol/min/mg protein) than at 35°C (0.028 µmol/min/mg protein). The lowest esterase activity was recorded at 37°C with 0.012 µmol/min/mg protein.

In case of drought tolerant isolate MC 2, mycelium produced in PDB without PEG (control) recorded significantly highest esterase activity (0.050 µmol/min/mg protein) followed by 20 per cent PEG with an esterase activity (0.024 µmol/min/mg protein). Lowest activity of 0.015 µmol esterase/min/mg protein was observed at 35 per cent PEG in the PDB medium (Table 31).

4.3.3. Activity of Antioxidant Enzyme, Catalase in the Stress Tolerant Isolates

Catalase in the mycelia of thermotolerant isolate MC 7 at temperatures of 27 (control), 35 and 37°C as well as drought tolerant isolate MC 2 at PEG concentrations of 20 and 35 per cent and control (without PEG) were analysed (Table 30 and 31).

Significantly higher catalase value of 0.140 EU/min/mg protein was recorded in the isolate MC 7 at 37ºC. Lowest catalase activity was registered at temperatures of 35°C and 27°C with values of 0.003 and 0.002 EU/min/mg protein respectively, being on par with each other.

Drought tolerant isolate MC 2 also recorded significantly highest catalase activity of 0.294 EU/min/mg protein at 35 per cent PEG compared to that of PEG concentration 20 per cent. Control achieved the lowest catalase activity of 0.005 EU/min/mg protein which was significantly lower than the catalase activity at higher concentrations of PEG.

4.3.4. Activity of Antioxidant Enzyme, Peroxidase in the Stress Tolerant Isolates

The peroxidase activity of thermotolerant mycelia (MC 7) was varied within a range of 0.001 to 0.058 EU/min/g tissue weight. Significantly highest peroxidase activity was registered in the isolate MC 7 at 37°C with an activity of 0.058 EU/min/g tissue weight followed by 35°C with 0.015 EU/min/g tissue weight and the lowest activity was reported at optimum temperature (27°C) with peroxidase activity of 0.001 EU/min/g tissue weight (Table 30).

Likewise, when the drought tolerant isolate MC 2, at concentrations of 20 and 35 per cent PEG in the PDB medium exhibited a peroxidase activity, varying from 0.004 to 0.030 EU/min/g tissue weight respectively (Table 31). Significantly highest activity was shown by the mycelium at 35 per cent PEG (0.030 EU/min/g tissue weight) in the PDB medium followed by mycelium at 20 per cent PEG (0.004 EU/min/g tissue weight). Lowest activity of 0.002 EU/min/g tissue weight was recorded in the control.

4.3.5. Trehalose Content in the Stress Tolerant Isolates of *Metarhizium anisopliae*

Trehalose content in the stress tolerant isolates (MC 2 and MC 7) was estimated and results are presented in the Table 30 and 31.

The highest trehalose content was observed in the temperature tolerant isolate MC 7, at 37ºC (1.380 mg/min/g mycelia). Trehalose content showed a reducing trend with decrease in temperature. At 35ºC, MC 7 recorded lower trehalose content of 1.270 mg/min/g mycelia. Only a less quantity of trehalose was estimated at temperature of 27ºC (0.760 mg/min/g mycelia).

Quantity of trehalose in the drought tolerant isolate MC 2 was significantly higher at highest concentration of 35 per cent PEG with a values of 1.580 mg/min/g mycelia followed by 20 per cent PEG concentration with a value of 1.151 g/min/g

mycelia. Lowest trehalose content of 0.668 mg/min/g mycelia was estimated in the control.

4.4. *IN VITRO* EVALUATION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* AGAINST *Tribolium castaneum*

Biocontrol potential of two stress tolerant isolates at different doses of 10^5 to 10^9 against the storage pest *Tribolium castaneum* was studied. The mortality caused to adults and grubs was recorded and the dose-mortality relationship was worked out for each isolate (Plate 13). *Metarhizium anisopliae* NBAIR strain was maintained as positive control. The results are given in the Table 32, 33, 34 and 35.

4.4.1. Effect of *Metarhizium anisopliae* Isolates on *Tribolium castaneum*

The effect of the selected isolates, namely MC 2, MC 4 and MC 7 was evaluated on adults and grubs through contact toxicity bioassay techniques. Number of insects killed at each concentrations was recorded at 24 h intervals for fifteen days. The results are presented in the Tables 32 and 33.

Six days after treatment, the isolates MC 2 and MC 7, applied at concentrations of 10^7 to 10^9 spores/ml caused adult mortality which ranged between 10.00 and 16.66 per cent. However, NBAIR strain, applied at 10^6 to 10^9 spores/ml induced mortality varying from 3.33 to 20 per cent. None of the isolates except positive control (NBAIR strain) recorded mortality at dose 10^6 spores/ml.

At nine days after treatment, isolate MC 2 and positive control recorded 40 and 46.66 per cent mortality when applied at 10^9 spores/ml. It was then immediately followed by the isolate MC 7 with 33.33 per cent mortality.

Twelve days after treatment, isolates MC 2, MC 7 and positive control induced more than 50 per cent mortality in adults when applied at 10^7 to 10^9 spores/ml. Isolate MC 2 when applied at 10^8 and 10^9 spores/ml caused mortality of

Table 30. Biochemical assay of thermotolerant isolate (MC 7) of *Metarhizium anisopliae*

Sl. No.	Temperature (ºC)	Total protein (mg/ml) *	Esterase (µmol/min/ mg protein) *	Catalase (EU/min/mg protein) *	Peroxidase (EU/min/g tissue weight) *	Trehalose content (mg/min/g mycelia) *
1.	Control (27)	4.410[b]	0.036[a]	0.002[b]	0.001[c]	0.760[c]
2.	35	4.600[b]	0.028[b]	0.003[b]	0.015[b]	1.270[b]
3.	37	5.721[a]	0.012[c]	0.140[a]	0.058[a]	1.380[a]
CD (0.05)		0.597	0.008	0.004	0.003	0.072

***** Mean of five replications

Table 31. Biochemical assay of drought tolerant isolate (MC 2) of *Metarhizium anisopliae*

Sl. No.	PEG (%)	Total protein (mg/ml) *	Esterase (µmol/min/ mg protein) *	Catalase (EU/min/mg protein) *	Peroxidase (EU/min/g tissue weight) *	Trehalose content (mg/min/g mycelia) *
1.	Control	5.020[a]	0.050[a]	0.005[c]	0.002[c]	0.668[c]
2.	20	4.270[b]	0.024[b]	0.083[b]	0.004[b]	1.151[b]
3.	35	2.831[c]	0.015[c]	0.294[a]	0.030[a]	1.580[a]
CD (0.05)		0.130	0.001	0.007	0.001	0.016

***** Mean of five replications

53.33 and 76.66 per cent respectively whereas positive control induced 73.33 and 83.33 per cent mortality of adult insects at the corresponding spore concentrations.

All the isolates caused mortality above 50 per cent in adult insects by 15^{th} day at all concentrations except in case of isolate MC 4 (Table 32). At the highest concentration of 10^9 spores/ml, isolate MC 2 and positive control caused 86.66 per cent mortality in adults whereas isolate MC 7 had caused 83.33 per cent mortality at the same dose. At lowest concentration of 10^5 spores/ml, isolate MC 2 and MC 7 recorded 53.33 and 50 per cent mortality whereas positive control induced 63.33 per cent mortality in adults.

Mortality of grubs by *M. anisopliae* isolates at different time intervals and concentrations were also observed (Table 33). All isolates recorded mortality in grubs at three days after treatment when applied at concentrations ranging from 10^7 to 10^9 spores/ml. Seven days after treatment, isolate MC 2 caused 83.33 and 100 per cent mortality of grubs at 10^8 and 10^9 spores/ml. At the same concentrations, NBAIR strain caused cent per cent mortality of grubs while the isolate MC 7 caused only 73.33 and 83.33 per cent mortality in the grubs. Isolate MC 2 resulted in 66.66 per cent mortality at the lowest dose of 10^5 spores/ml which was identical with that of positive check (63.33 %), while MC 7 caused only 43.33 per cent mortality.

Twelve days after treatment, cent per cent mortality of grubs were observed by the isolate MC 2 and positive check when applied at 10^5 spores/ml. MC 7, caused 93.33 per cent mortality in grubs for the corresponding period while MC 4 caused the lowest mortality of 50 per cent. All the isolates caused cent per cent mortality at fifteen days after treatment at all doses evaluated except MC 4 applied at 10^5 spores/ml, which recorded 93.33 per cent mortality. By 15^{th} day of treatment, the same dose caused 100 per cent mortality in the isolate MC 7 and isolate MC 4 was recorded with a least mortality rate of 93.33 per cent.

4.4.2. Dose-Mortality Responses of Adults and Grubs of *Tribolium castaneum*

Lethal time required to cause 50 (LT_{50}) and 90 (LT_{90}) per cent mortality in

adults was worked out for all the isolates. Isolate MC 2 had the lowest LT_{50} and LT_{90} values of 9.43 and 16.01 days respectively at the highest concentration of 10^9 spores/ml (Table 34). This was identical with positive control which took 9.06 and 15.45 days to kill 50 and 90 per cent population respectively at the same dose. The isolate MC 7, required 10.07 and 16.42 days to kill 50 and 90 per cent population of adult insects. At 10^8 spores/ml, LT_{50} and LT_{90} values for the isolate MC 2 was about 10.63 and 19.58 days while MC 7 took 11 and 19.70 days for mortality of adults which was found on par with positive control with a LT_{50} and LT_{90} values of 9.98 and 16.95 days.

At the lowest concentration of 10^5 spores/ml, LT_{50} and LT_{90} of the isolate MC 7 was low (15.12 and 23.32 days) when compared to that of MC 2 (15.16 and 24.66 days). At the same time, positive control recorded LT_{50} and LT_{90} values of 14.22 and 22.68 days.

In the bioassay using grubs (Table 35), *M. anisopliae* NBAIR strain had the lowest LT_{50} and LT_{90} values of 4.13 and 6.38 days at 10^9 spores/ml. Similar results were also recorded by the isolate MC 2 with values of 4.14 and 6.58 days at the same concentration. LT_{50} and LT_{90} values for the isolate MC 7 were 4.62 and 7.67 days. At 10^8 spores/ml, lowest LT_{50} and LT_{90} were registered by the isolate MC 2 with a values of 4.62 and 7.66 days which was found comparable to that of NBAIR strain with a LT_{50} and LT_{90} values of 4.54 and 7.40 days respectively. At the same time isolate MC 7 recorded LT_{50} and LT_{90} values of 5.17 and 8.13 days.

When the isolates were applied at the lowest concentration of 10^5 spores/ml, isolate MC 2 recorded LT_{50} and LT_{90} of 6.09 and 9.27 days whereas in positive control, LT_{50} and LT_{90} values were about 7.14 and 9.54 days being on par with each other. From the study, it was found that there was no significant difference exhibited in the biocontrol efficacy among the stress tolerant isolates when compared with that of positive control (NBAIR strain).

(i) MC 2 (adult)

(ii) MC 2 (grub)

(i) MC 4 (adult)

(ii) MC 4 (grub)

(i) MC 7 (adult)

(ii) MC 7 (grub)

Plate 13. Adults and grubs of *Tribolium castaneum* infected by *Metarhizium anisopliae* isolates

Table 32. Mortality of adult insects (%) caused by *Metarhizium anisopliae* isolates

M. anisopliae isolates	Dose (cfu ml^{-1})	Mortality of adult insects (%) *			
		6th day	9th day	12th day	15th day
MC 2	1×10^5	-	16.66	30.00	53.33
	1×10^6	-	23.33	33.33	56.66
	1×10^7	10.00	26.66	46.66	73.33
	1×10^8	13.33	36.66	53.33	76.66
	1×10^9	16.66	40.00	76.66	86.66
MC 4	1×10^5	-	-	20.00	36.66
	1×10^6	-	13.33	23.33	43.33
	1×10^7	-	16.66	36.66	63.33
	1×10^8	6.66	16.66	43.33	66.66
	1×10^9	10.00	26.66	56.66	70.00
MC 7	1×10^5	-	6.66	26.66	50.00
	1×10^6	-	13.33	33.33	53.33
	1×10^7	10.00	20.00	43.33	73.33
	1×10^8	13.33	26.66	50.00	80.00
	1×10^9	13.33	33.33	73.33	83.33
NBAIR strain (Postivie control)	1×10^5	-	13.33	33.33	63.33
	1×10^6	3.33	16.66	36.66	66.66
	1×10^7	10.00	23.33	50.00	73.33
	1×10^8	13.33	36.66	73.33	83.33
	1×10^9	20.00	46.66	83.33	86.66

***** Mean of three replications

Table 33. Mortality of grubs (%) caused by *Metarhizium anisopliae* isolates

M. anisopliae isolates	Dose (cfu ml^{-1})	Mortality of grubs (%)*			
		3rd day	7th day	12th day	15th day
MC 2	1×10^5	-	66.66	100.00	100.00
	1×10^6	-	73.33	100.00	100.00
	1×10^7	13.33	76.66	100.00	100.00
	1×10^8	20.00	83.33	100.00	100.00
	1×10^9	23.33	100.00	100.00	100.00
MC 4	1×10^5	-	33.33	50.00	93.33
	1×10^6	-	43.33	53.33	100.00
	1×10^7	-	53.33	100.00	100.00
	1×10^8	10.00	56.66	100.00	100.00
	1×10^9	16.66	63.33	100.00	100.00
MC 7	1×10^5	-	43.33	93.33	100.00
	1×10^6	-	60.00	100.00	100.00
	1×10^7	10.00	66.66	100.00	100.00
	1×10^8	16.66	73.33	100.00	100.00
	1×10^9	20.00	83.33	100.00	100.00
NBAIR strain (Positive control)	1×10^5	-	63.33	100.00	100.00
	1×10^6	-	76.66	100.00	100.00
	1×10^7	13.33	83.33	100.00	100.00
	1×10^8	16.66	100.00	100.00	100.00
	1×10^9	23.33	100.00	100.00	100.00

***** Mean of three replications

Table 34. Dose-mortality response of adult insects of *Tribolium castaneum* with *Metarhizium anisopliae* isolates

Dose (cfu/ml)	*M. anisopliae* isolates	LT_{50} (Days)	Fiducial limit (95%)	Chi-square	Slope	LT_{90} (Days)	Fiducial limit (95%)
1×10^5	MC 2	15.16	13.76-17.89	4.87	5.23	24.66	20.41-34.58
	MC 4	16.40	14.91-19.74	3.22	7.29	26.58	21.23-37.34
	MC 7	15.12	13.97-17.28	3.40	6.82	23.32	19.66-32.19
	NBAIR strain	14.22	13.21-15.91	4.06	6.32	22.68	19.30-30.18
1×10^6	MC 2	14.53	13.14-17.21	3.68	4.52	27.91	21.91-44.62
	MC 4	15.71	14.31-18.50	4.39	6.22	25.24	20.70-37.25
	MC 7	14.41	13.26-16.41	3.90	5.65	24.28	20.16-34.05
	NBAIR strain	13.40	12.44-14.85	3.71	5.68	22.52	19.13-29.74
1×10^7	MC 2	12.10	11.20-13.41	1.81	4.61	22.96	19.06-31.86
	MC 4	13.58	12.66-15.00	3.79	6.12	21.98	18.83-28.66
	MC 7	12.26	11.37-13.55	2.55	4.84	22.56	18.90-30.61
	NBAIR strain	11.42	10.69-12.33	2.18	5.40	19.74	17.17-24.72
1×10^8	MC 2	10.63	9.88-11.49	1.44	4.83	19.58	16.86-25.13
	MC 4	13.16	12.15-14.75	1.51	4.50	23.76	19.73-32.93
	MC 7	11.00	10.26-11.89	2.65	5.07	19.70	17.03-25
	NBAIR strain	9.98	9.33-10.64	1.57	5.57	16.95	15.12-20.20
1×10^9	MC 2	9.43	8.78-10.05	2.32	5.57	16.01	14.38-18.87
	MC 4	12.00	11.15-13.16	2.67	4.95	21.77	18.43-28.88
	MC 7	10.07	9.47-10.70	3.10	6.03	16.42	14.82-19.16
	NBAIR strain	9.06	8.40-9.67	5.10	5.53	15.45	13.91-18.10

***** Mean of three replications

Table 35. Dose- mortality responses of grubs of *Tribolium castaneum* with *Metarhizium anisopliae* isolates

Dose (cfu/ml)	*M. anisopliae* isolates	LT_{50} (Days)	Fiducial limit (95%)	Chi-square	Slope	LT_{90} (Days)	Fiducial limit (95%)
1×10^5	MC 2	6.09	5.69-6.49	5.42	7.01	9.27	8.54-10.37
	MC 4	9.54	8.67-10.54	14.56	4.50	18.38	15.57-23.99
	MC 7	6.85	6.41-7.29	6.22	6.55	10.76	9.87-12.10
	NBAIR strain	7.14	5.57-6.73	7.04	6.70	9.54	8.44-11.61
1×10^6	MC 2	5.52	4.91-6.13	6.80	7.69	8.38	7.33-10.67
	MC 4	8.40	7.53-9.29	18.13	4.77	15.57	13.37-19.84
	MC 7	6.32	5.72-6.94	7.43	6.66	9.84	8.65-12.24
	NBAIR strain	5.68	5.13-6.21	7.19	6.96	8.69	7.75-10.42
1×10^7	MC 2	5.03	4.40-5.67	6.94	5.92	8.27	5.92-6.77
	MC 4	6.63	5.92-7.38	9.49	5.38	11.47	9.84-14.94
	MC 7	5.67	5.21-6.13	5.73	5.50	9.70	8.66-11.43
	NBAIR strain	5.04	4.18-5.94	7.90	6.49	7.93	6.55-13.05
1×10^8	MC 2	4.62	3.90-5.28	4.98	5.83	7.66	6.46-11.03
	MC 4	5.77	5.15-6.40	6.49	5.34	10.02	8.65-12.83
	MC 7	5.17	4.44-5.87	7.00	5.51	8.83	7.44-12.35
	NBAIR strain	4.54	3.71-5.35	3.55	6.05	7.40	6.52-13.27
1×10^9	MC 2	4.14	2.97-5.02	5.36	6.38	6.58	5.34-14.46
	MC 4	5.14	4.28-6.97	8.15	5.08	9.19	7.54-15.29
	MC 7	4.62	3.76-5.38	6.44	5.82	7.67	6.34-12.16
	NBAIR strain	4.13	3.17-4.89	4.66	6.77	6.38	5.30-11.33

***** Mean of three replications

Discussion

5. DISCUSSION

The entomopathogenic fungi, *Metarhizium anisopliae* is a widely exploited biocontrol agent in the field of pest management. The genus *Metarhizium* inhabit the soil as saprobes, as rhizosphere inhabitants (Hu and St. Leger, 2002), as endophytes (Behie *et al*., 2012; Wyrebek *et al*., 2011) and also show a complex symbioses as pathogens of insects (Vega *et al*., 2009) and antagonism of fungal plant pathogens (Sasan and Bidochka, 2012). It is a mitosporic fungus with an anamorphic stage belonging to Order Hypocreales under division Ascomycota.

Various abiotic factors affected the sustainability of fungi and the products of *M. anisopliae* available in the markets are only suitable for general conditions. Hence, there is a need to identify the potential abiotic stress tolerant isolates of *M. anisopliae* that can survive under adverse environmental conditions and also having a good biocontrol efficacy against pests. The discussions on the experiments are presented in this chapter.

5.1. COLLECTION OF SOIL SAMPLES AND ISOLATION OF *Metarhizium anisopliae* ISOLATES

5.1.1. Physico-Chemical Properties of Soil Samples Collected from Stressed Locations of Kerala

A total of 40 soil samples were collected from different locations (stressed areas) of five districts and the soil temperature, moisture content, pH and EC of the collected soil samples were analysed prior to isolation. Domsch *et al*. (1980), Gal-Hemed *et al*. (2011) and Poosapati *et al*. (2014) suggested that the soil chemical properties like soil reaction and electrical conductivity had influenced diversity and existance of microbes in soil.

Temperature of the soil samples from various locations in Thrissur ranged between 40.00 and 43.08ºC, while soil moisture varied from of 1.20 to 4.00 per cent. The soils were moderately to strongly acidic, with pH of 4.40 to 5.40. The salinity, as indicated an EC values ranged from 0.01 to 0.02 dS m^{-1}.

Soils from various locations in Palakkad district recorded temperature between 44 and 46ºC, which was the highest among all samples. Soil moisture as well as EC were in the range of 1.10 to 2.70 and 0.01 to 0.02 dS m^{-1} respectively. pH values of 5.20 to 6.00 indicated slight to moderate levels of acidity.

Soil samples from locations in Ernakulam district showed a temperature range of 36.00 to 37.70ºC, soil moisture of 23.30 to 28.30 per cent and EC of 4.10 to 4.40 dS m^{-1}. The soils were highly acidic with pH values of 4.20 to 4.61.

Soils from Idukki district recorded comparatively lower temperature ranging from 29 to 31.50ºC, soil moisture varied from 5.90 to 7.70 per cent, pH was between 4.10 and 5.81 showing moderately to strongly acidic reaction and EC lay within a range of 0.41 to 0.91 dS m^{-1}.

Strongly acidic soils of Alappuzha district (pH 3.45 - 3.90) recorded soil temperature in the range of 37.70 to 38.50ºC, soil moisture between 34.50 and 40 per cent and EC between 5.22 and 6.68 dS m^{-1}.

The above values were identical to those reported by Bastin *et al*. (2014) who recorded pH of soil samples within a range of 5.58 to 8.58 from Palakkad, 3.91 to 7.90 from Alappuzha and 3.50 to 7.40 from Thrissur. Kavitha and Sujatha (2015) also reported that Thrissur soils were having a pH of 2.90 to 7.70. Indira and Covilakom (2013) pointed out that Kuttanad tracts of Alappuzha had soil pH between 2.50 and 5.20. Mohan and Sreelatha (2016) observed pH of Vyttila soils which varied between 3.90 and 4.26, similar to the findings of the present study. According to Koruth *et al*. (2014), most of the Kerala soil were acidic in reaction with a pH range of 3.00 to 6.80.

Bastin *et al.* (2014) estimated an EC value of 0.16 dS m^{-1} for soil samples of Alappuzha, 0.03 to 0.85 dS $m^{-1}$from Palakkad and 0.06 dS m^{-1} from Thrissur which could be comparable with the present study.

5.1.2. Isolation and Enumeration of *Metarhizium* spp.

Metarhizium anisopliae was isolated from the soil samples collected from different locations of Kerala following serial dilution pour plating method as well as bait trap method.

Isolates of *M. anisopliae* were obtained only from Alappuzha out of the four districts surveyed. A total of five isolates was obtained from Moncompu in Alappuzha district. In the present study, population of the fungus was highest in the soil collected from Moncompu 7 (19.66×10^3 cfu g^{-1} and 7.33×10^4cfu g^{-1}), least in soil of Moncompu 2 (13.33×10^3 cfu g^{-1} and 4.66×10^4 cfu g^{-1}) (Fig 2 and 3). According to Imoulan and Alaoui (2011) in Argan forest of Morocco as well as by Ekesi *et al*. (2005) in Kenya, *Metarhizium anisopliae* populations occur at comparatively lower levels than those of other entomopathogenic fungi like *B. bassiana*. The later had enumerated *M. anisopliae* population, to be ranging from 1.9 to 3×10^5 cfu/g in sandy loam soils with pH of 4.7.

5.1.2.1. Characterization of Metarhizium anisopliae Isolates

Cultural, morphological and molecular characterization of *M. anisopliae* isolates from different locations were carried out, narrowing the different populations to three isolates of *M. anisopliae*, depicted as MC 2, MC 4 and MC 7. Isolate MC 2 had offwhite mycelial growth with light olive green conidia while MC 4 produced green coloured mycelial growth with green conidia. MC 7 also exhibited uniformly green mycelial growth but had dark olive green conidia. This is in conformity with the findings of Ghayedi and Abdollahi (2013), Bridge *et al*. (1993) and Sepulveda *et al*. (2016) who have reported that *M. anisopliae* colonies grown in PDA medium

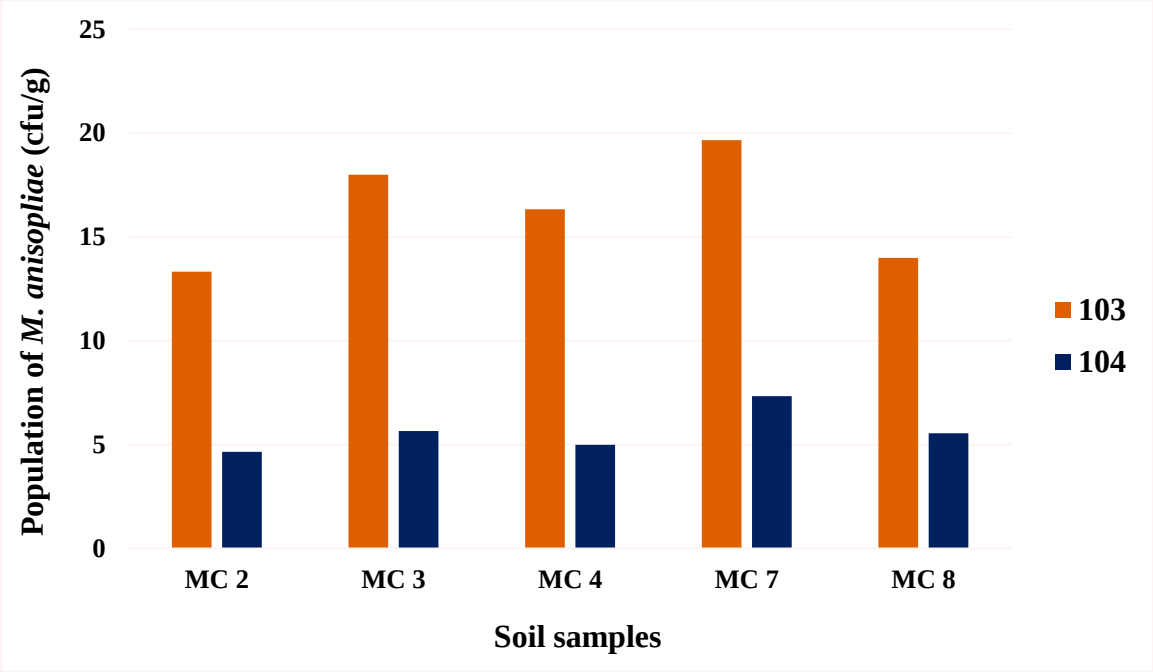


Fig 2. Enumeration of *Metarhizium* spp. in Veen's selective medium

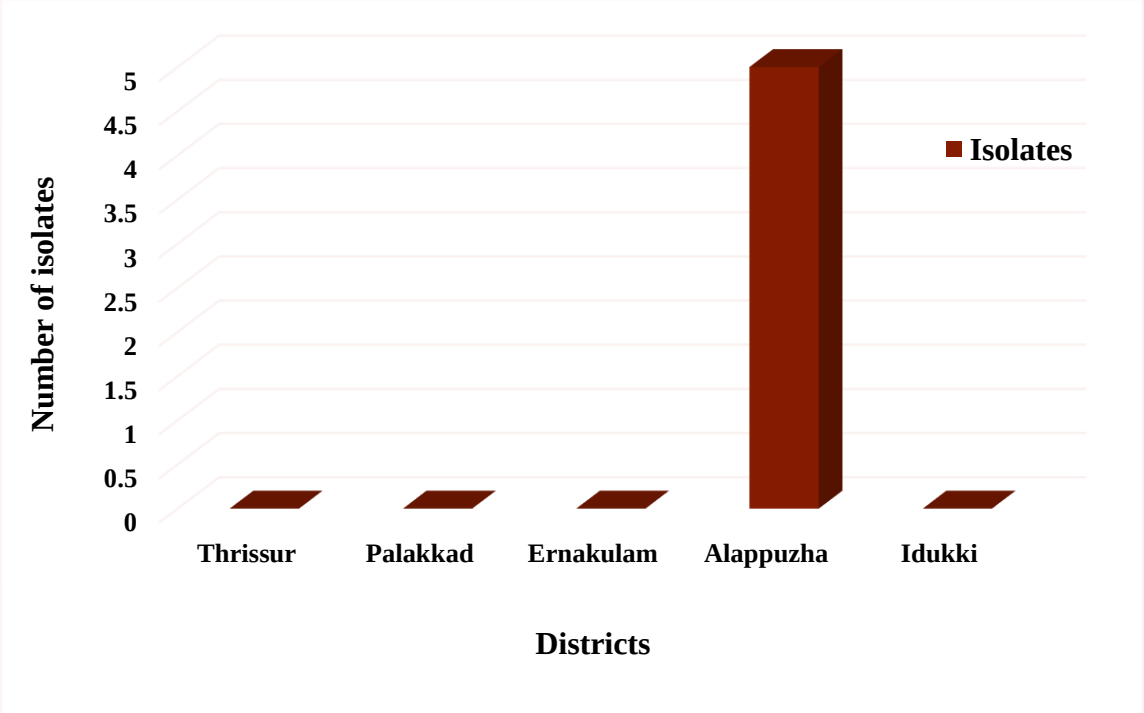


Fig 3. Number of *Metarhizium anisopliae* isolates obtained from districts

produced olivaceous, yellowish green and dark green conidia. Similar findings were also made by Fernandes *et al*. (2010) and Brunner-Mendoza *et al*. (2019) where they reported that *M. anisopliae* grown in PDA medium showed dark green, light green or brownish or even bicoloured growth. They have also observed that the colour of the mycellial growth on the reverse side of the plate as orangish yellow to white. Similarly, Tangthirasunun *et al*. (2010) reported the cultural characters of 24 *M. anisopliae* isolates from Thailand grown in PDA medium and according to him, the mycelium was initially white or creamy in colour which later turned into shades of yellow, green or dark green during sporulation. .

Morphometric studies of the three isolates showed that MC 2 formed conidia with 3.53 µm in mean length and 1.61 µm in mean width, phialides with mean length of 6.61 µm and mean width of 1.51 µm. The dimensions of conidia were 3.72 µm × 1.38 µm and that of phialides were 4.72 µm × 1.32 µm in MC 4. MC 7 produced conidia of 4.05 µm × 1.70 µm and phialides of 7.01 µm × 1.50 µm. Rombach *et al*. (1986) and Tulloch (1976) had reported that conidial length of *M. anisopliae* var. *anisopliae* was between 3.5 and 9.0 µm. Liu *et al*. (2012), who characterized *M. anisopliae* var. *anisopliae* grown in potato dextrose + peptone agar (1 %) medium had recorded that as cylindrical with obtuse end and the conidia had a mean length of 4 to 8 µm and mean width of 1.5 to 3.0 µm. Bridge *et al*. (1993) noticed the morphological characters like conidial length and width of 24 isolates of *M. anisopliae* grown in potato carrot agar and found the conidial length ranging between 4.5 and 8.4 µm and width between 1.6 and 3.2 µm.

The shape of conidia was cylindrical dome for all isolates. The arrangement of conidia originated as chains from tip of phialides except in case of MC 4 where conidia appeared as clusters as well as in chains. These observations are in agreement with those of Samson *et al*. (1988) and Sepulveda *et al*. (2016) who observed the conidia as cylindrical with round edges, arranged in chains from phialides. Similar findings were also obtained by Glare *et al*. (1996) where they observed the

morphology of phialides of 11 isolates of *M. anisopliae* and *M. flavoviride* grown in potato dextrose agar medium and concluded that most of the phialides were clavate to cylindrical with length ranging from 4.2 to 15.3 µm and width of 1.5 to 3.2 µm in case of *M. anisopliae*. The authors concluded that the phialide morphology was not a reliable taxonomical criterion as the isolate could vary within the same culture as well as between different cultures.

5.2. *IN VITRO* SCREENING OF *Metarhizium anisopliae* ISOLATES FOR STRESS TOLERANCE

Screening of isolates of *M. anisopliae* for tolerance to high temperature, salinity, acidity, drought, insecticides and fungicides was carried out. The tolerant isolates thus obtained can be manipulated and utilized as an effective biocontrol agent in the field of integrated pest management.

5.2.1. Screening of *Metarhizium anisopliae* Isolates for Acidity Tolerance

Isolates of *M. anisopliae* were grown in the PDB adjusted to four different levels of pH such as 5.5, 4.5, 3.5, 3 and 2.5, in order to identify the most acidity tolerant isolate among the three. There was a significant difference in the mycelial weights of isolates at different pH levels (Fig 4). In general, a reduction in mycelial biomass of isolates was observed when exposed to increasing levels of acidity.

At pH 6, highest mycelial biomass of 5.63 g was produced by the isolate MC 7 and the lowest by the isolate MC 2 with 5.46 g. The isolate MC 7 consistently produced highest biomass at all pH levels and was significantly superior to both MC 4 and MC 2. While the isolate MC 2 produced the lowest amount of biomass irrespective of pH levels, with mycelial mass ranging from 5.46 g at pH of 6.0 to 0.86 g at pH 2.5. Isolate MC 4 had the mycelial weight that was intermediate between the above isolates.

Sporulation by the different isolates were influenced by acidity and decreased at lower pH levels. The three isolates showed high to medium sporulation at pH of 6.0 to 4.5. Isolate MC 7 demonstrated highest sporulation at pH of 3.5 and medium sporulation at pH 3. Based on mycelial production as well as sporulation, MC 7 appeared to be the most tolerant to acidity among all the isolates evaluated. This suggests that the isolate MC 7 could be a better choice for biocontrol in highly acidic soils as encountered in Kuttanad. The results from the study were in accordance with the study of Teja and Rahman (2017) as they had observed highest fungal biomass production by *M. anisopliae* isolates at pH values from 4 to 6. According to Hallsworth and Magan (1996), the optimal pH levels for the growth of entomopathogenic fungi was between 5 and 8. This implies that most of the entomopathogenic fungi can regulate cytosolic pH more effectively than many other microbes. The ability of entomopathogens to grow well at pH below 7 might be a desirable during industrial production, enable reduction in the pH of the substrate so as to inhibit the growth of contaminants without affecting the yield.

Kotwal *et al*. (2012) reported that *M. anisopliae* showed highest radial growth of 35.66 mm at pH 5 in Sabauraud's dextrose yeast agar medium attaining complete sporulation by 15^{th} day. However, Namasivayam *et al*. (2015) reported highest *Metarhizium* fungal colony count of 77.2×10^8 per gram of soil at pH 7 as against colony count of 4.2×10^8 per gram of soil at pH 5.5. The present study has also shown that *M. anisopliae* favours pH values above 3.5 for optimal growth and sporulation.

5.2.2. Screening of *Metarhizium anisopliae* Isolates for Salinity Tolerance

The three isolates of *M. anisopliae* were screened for their response to various salinity levels so as to identify the isolate that could survive under high osmotic pressure (Fig 5).

In the present study, it was observed that the growth and sporulation of all isolates decreased as the concentration of NaCl increased. Lowest growth inhibition was observed for the isolate MC 2 at all salinity levels tested with growth inhibition of 6.33 per cent at 0.5 M NaCl concentration and 94.10 per cent at the highest NaCl concentration of 2 M. At the same time, the isolate MC 7 recorded highest degree of growth inhibition, ranging from 54.71 per cent at 0.5 M NaCl to cent per cent at 2 M NaCl concentrations. Isolate MC 4 also recorded higher growth inhibition of 50.32 per cent to cent per cent at same respective concentrations. The sporulation of all isolates were also adversely affected by higher salinity levels. MC 2 showed moderate sporulation at a concentration of 0.5 M and sparse sporulation at 1 M while other two isolates didn't show any sporulation at 1 M and above.

Records of Rangel *et al*. (2008) was in contrary with the present study. However, they isolated a strain ARSEF 25 of *M. anisopliae* that sporulated at salt concentration of 0.8 M. The conidia so produced were more virulent than those produced under normal salinity levels. In addition, they also observed that as salt concentration increased, might decreased the heat tolerance of conidia. This was in agreement with the present findings where the drought tolerant isolate MC 2 could not withstand higher temperatures.

5.2.3. Screening of *Metarhizium anisopliae* Isolates for Temperature Totolerance

Temperature is one of the most important environmental factor as far as growth and sporulation of fungus are concerned. The ability of the entomopathogens to survive under a wide range of temperatures is critical for their success in the field and it has been employed as a criterion for screening of the isolates as biocontrol agent. Isolates obtained from different locations were screened at temperatures of 25, 27, 30, 35, 37 and 40ºC in order to identify isolate which could survive at high temperatures.

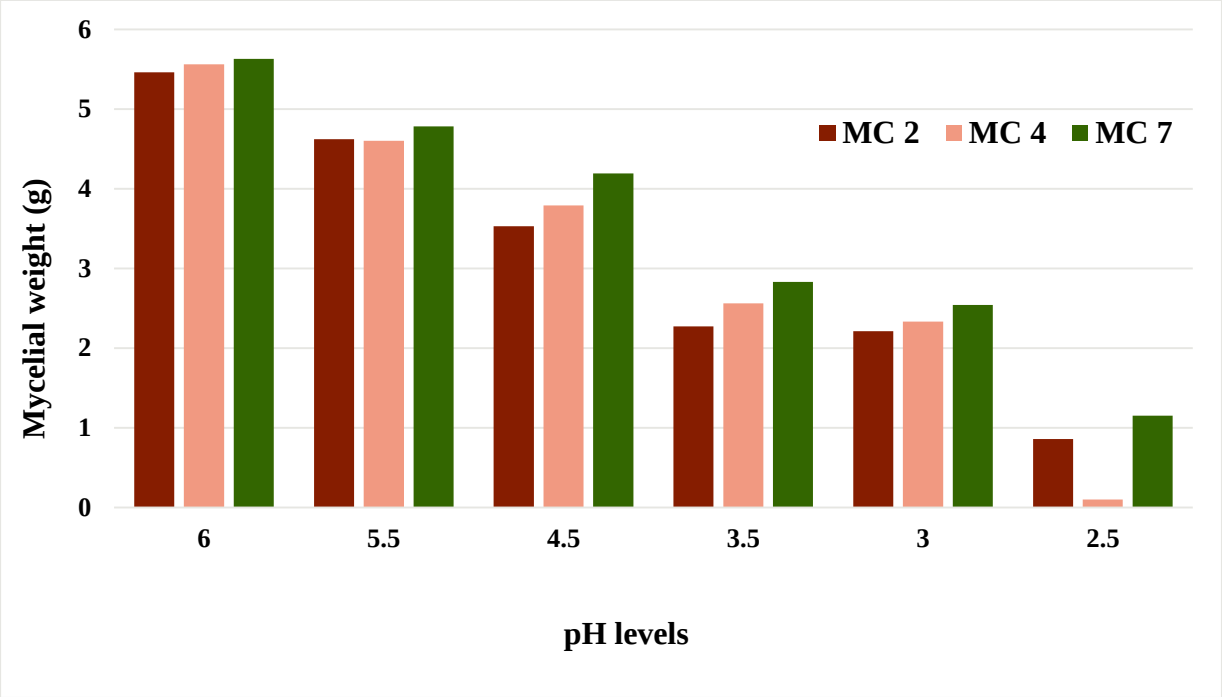


Fig 4. Effect of pH on the growth of *Metarhizium anisopliae* isolates

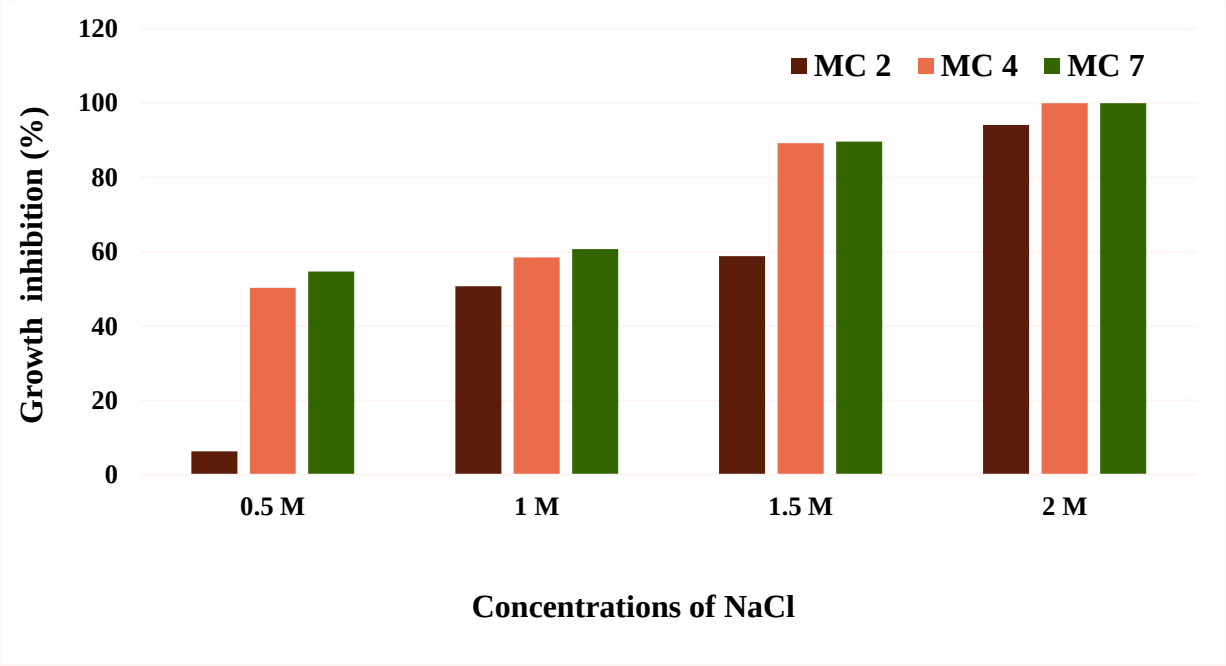


Fig 5. Effect of NaCl on the growth of *Metarhizium anisopliae* isolates

The isolate MC 7 produced significantly higher mycelia at all temperatures evaluated than other isolates except at 27ºC where it was on par with the isolate MC 4. The isolate, MC 2 with biomass of 5.46 g at 27ºC and 0.38 g at 37ºC produced the least amount of mycelia at all temperatures (Fig 6). Observations on sporulation also followed the above pattern. While MC 7 showed high to medium sporulation upto 30ºC and sparse sporulation at 37ºC, other isolates sporulated sparsely at 30ºC and did not sporulate at higher temperatures. On the whole, all isolates showed reduction in growth as well as sporulation at higher temperatures with the effect being most pronounced in case of MC 2 and MC 4.

Among the three isolates tested, MC 7 was the thermotolerant isolate which could survive at 37^0C while all other isolates could not survive at 37ºC. None of the isolates survived when they were exposed to at 40^0C. This was in conformity with the reports of Hallsworth and Magan (1999) and Teja and Rahman (2016) who pointed out that the upper limit of temperature for the growth and sporulation of *M. anisopliae* is from 37 to 40ºC. Again, Teja and Rahman (2016), who screened four isolates of *M. anisopliae* for thermotolerance at 25, 30, 35 and 40ºC, found that none of the isolates thrived at 40ºC. According to Dimbi *et al*. (2004) and Yewale (2001) optimum temperature for growth and sporulation of *M. anisopliae* was at 25ºC, while in the present study, the optimum temperature was at 27ºC. The experiments conducted by Ekesi *et al*. (2003) also reported the influence of soil temperature on the survival and infectivity of *M. anisopliae* isolates and found that mortality of the pest by all isolates were noticed at temperature between 20 and 30ºC. Dimbi *et al*. (2004) and Faria *et al*. (2015) studied the effect of temperature on germination, growth and virulence of *M. anisopliae* and found that temperature variations might have triggered physiological changes leading to the synthesis of heat shock proteins. As an increase in temperature had resulted in the increase in infectivity and virulence of *M. anisopliae* till the optimum temperature of that isolate is reached. In the study of

Samuels *et al*. (1989), postulated a positive relation between the conidial germination and virulence of EPF with respect to the effect of temperature towards target pest.

5.2.4. Screening of *Metarhizium anisopliae* Isolates for Drought Tolerance

Moisture is an important factor that influence the ability of entomopathogens to survive, propagate, infect and kill their host. So the present study was proposed to screen native tolerant isolates of *M. anisopliae* that can survive under drought situations.

The isolate MC 2 showed consistently higher tolerance to higher concentrations of PEG with mycelial mass of 2.39 g at 10 per cent PEG to 0.93 g at 35 per cent PEG. The isolate, MC 4 registered lowest biomass production among the isolates tested and MC 7 recorded intermittent values. Growth of the fungi as well as sporulation was adversely affected at higher PEG concentrations (Fig 7). Sparse to medium sporulation was exhibited by all the isolates at 20 per cent PEG concentration and at 30 per cent PEG, MC 2 alone produced sparse sporulation. Hence, isolate MC 2 was adjudged as the most drought tolerant among the three isolates.

The results of the present study was in confirmity with the findings of Chen *et al*. (2014) where they reported that an isolate of *M. anisopliae* namely MAX-2, recorded growth under dessication stress with greater efficacy against *Tenebrio molitor* larvae at moisture levels below 30 per cent. Similarly, Matawele *et al*. (1994) isolated a low water active, UV-mutant strain of *M. anisopliae* which showed good germination and was more virulent in controlling green leafhoppers than wild type strains. Hallsworth and Magan (1995) had manipulated the culture media in order to enhance the germination of conidia under moisture stress. The cultures were modified with glycerol and trehalose and effect of water stress was recorded at different water activity adjusted in the media. It was observed that conidia in the media with glycerol and trehalose germinated quickly and were more pathogenic at low water activity.

Likewise, Magan (2001) reported that xerophilic fungi are known to produce glycerols and polyols under water stress which enable them to survive under extreme conditions of environmental stresses. They also noticed that entomopathogens did not showed any conidial germination at below water activity of 0.92. Borisade and Magan (2014) found that out of five isolates of *M. anisopliae* tested, two isolates tolerated extreme water stress of 0.94 a_w.

5.2.5. Screening of *Metarhizium anisopliae* Isolates for Insecticide and Fungicide Tolerance

Combined application of insecticide or fungicide and entomopathogenic fungi provide satisfactory control against many agricultural insect pest. But use of incompatible pesticides or pesticide residues in soil could hinder the growth and development of fungi. In this context, experiment was conducted in order to screen the isolates of *M. anisopliae* for tolerance to different insecticides and fungicides.

In general, growth of all isolates reduced at higher doses of insecticides and fungicides (Fig 8 to 13). In the case of PDA amended with spinosad at different doses, all the isolates showed a growth inhibition of less than 19.50 per cent. Isolate MC 7 was the least inhibited with inhibition ranging from 8.11 to 11.41 per cent at higher doses of spinosad. The extent of inhibition increased as the dose of spinosad increased in the medium. Sporulation was adversely affected only at higher doses of insecticide. Isolate, MC 7 produced medium sporulation even at highest dose of spinosad. All the isolates *viz* MC 2, MC 4 and MC7 tolerated all the doses of spinosad tested and are considered compatible to spinosad (Fig 8).

Cypermethrin on the other hand, caused inhibition more than 50 per cent in all isolates even at the lowest dose (Fig 9). All the isolates were equally incompatible with cypermethrin, suggesting that combined application of cypermethrin and *M. anisopliae* should not be advisable. Compatibility of four isolates of *M. anisopliae* from Punjab and Pakistan with a number of pesticides had been reported by Akbar *et*

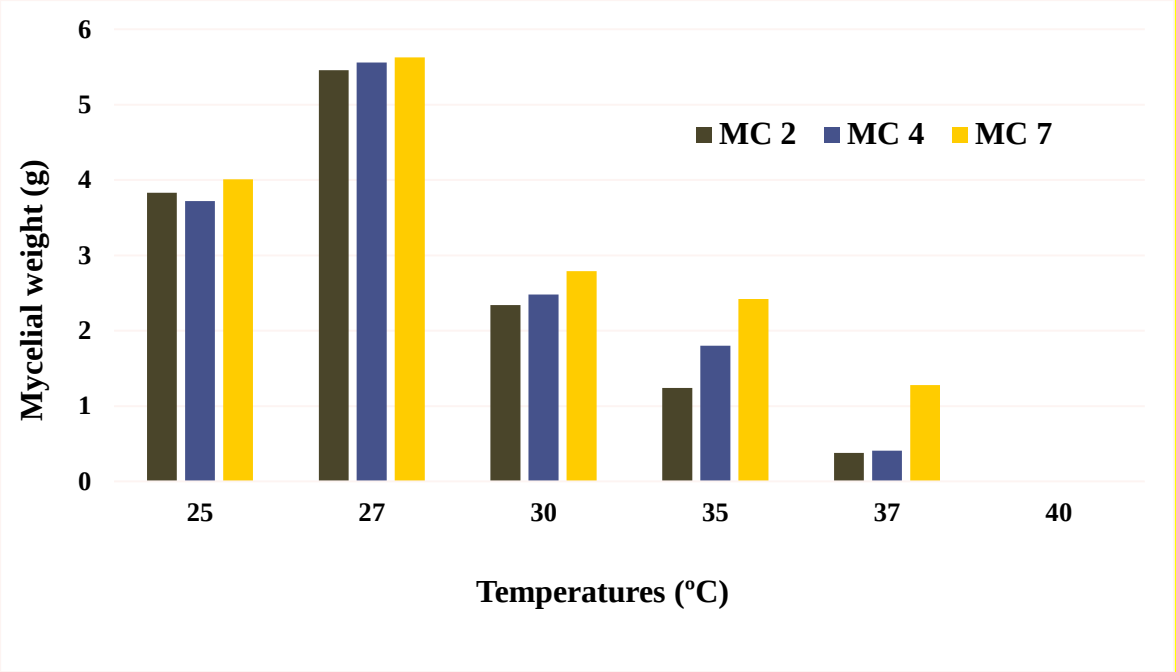


Fig 6. Effect of temperature on the growth of *Metarhizium anisopliae* isolates

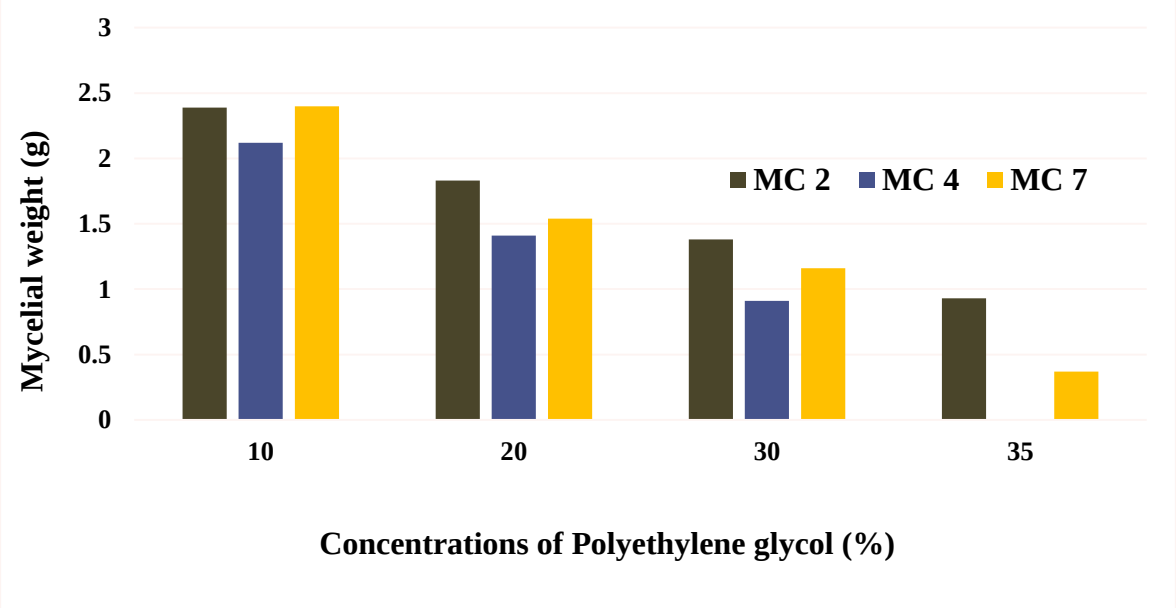


Fig 7. Effect of PEG on the growth of *Metarhizium anisopliae* isolates

al. (2012). The isolate M70 recorded highest radial growth of 6.81 cm and a spore yield of 1.26×10^8/ml in PDA amended with recommended dose of spinosad whereas imidacloprid, indoxacarb, cypermethrin, acetamiprid supported only moderate conidial germination. The study concluded that insecticides like spinosad, imidacloprid and acetamiprid were more compatible with *M. anisopliae* than other insecticides tested. The study concluded that insecticides like spinosad, imidacloprid and acetamiprid were more compatible with *M. anisopliae* than other insecticides tested.

The isolates had less than 22 per cent growth inhibition at all doses of imidacloprid, with growth inhibition of isolates ranging between 7.10 and 21.50 per cent and are considered compatible to imidacloprid (Fig 10). The isolate MC 7 was consistently superior to other isolates in terms of growth and sporulation. The inhibition was dose dependent for all isolates, with highest degree of inhibition being at the highest dose of the insecticide (0.15 g/L). This is in tune with the results of Mochi *et al*. (2005) where they mentioned that the insecticide, imidacloprid had no effect on the survival and growth of *M. anisopliae*. Imidacloprid had been reported as compatible with *M. anisopliae* by several authors. Quintela and McCoy (1998) reported that combined application of *M. anisopliae* and imidacloprid resulted in higher mortality of root weevil grub, *Diaprepes* sp. in soil. Moreover in the study of Neves *et al*. (2001) also confirmed compatibility of imidacloprid with *M. anisopliae*. But imidacloprid was found moderately toxic at maximum dose and incompatible at minimum dose with entomopathogens as stated by Filho *et al*. (2012).

The three isolates were also found to be compatible with chlorantraniliprole at all doses used in this study. The growth inhibition caused was less than nine per cent for all isolates at all doses (Fig 11). Significantly superior radial growth along with high sporulation at higher doses was exhibited by the isolate MC 7 when compared to other isolates.

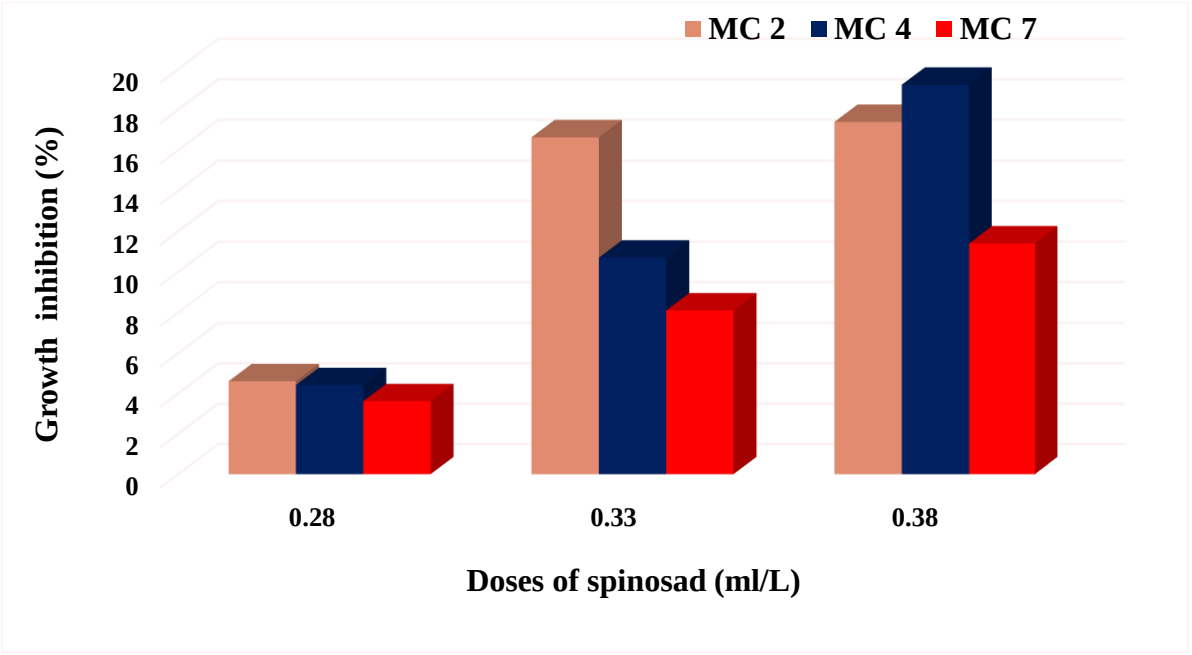


Fig 8. Effect of spinosad on the growth of *Metarhizium anisopliae* isolates

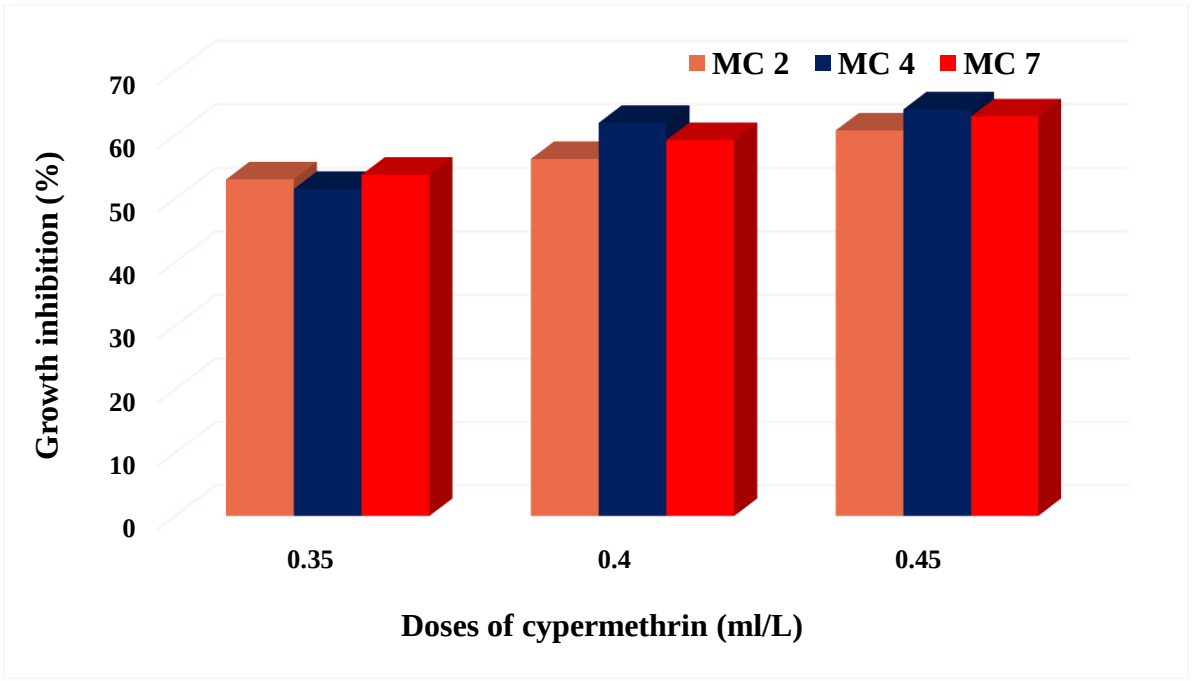


Fig 9. Effect of cypermethrin on the growth of *Metarhizium anisopliae* isolates

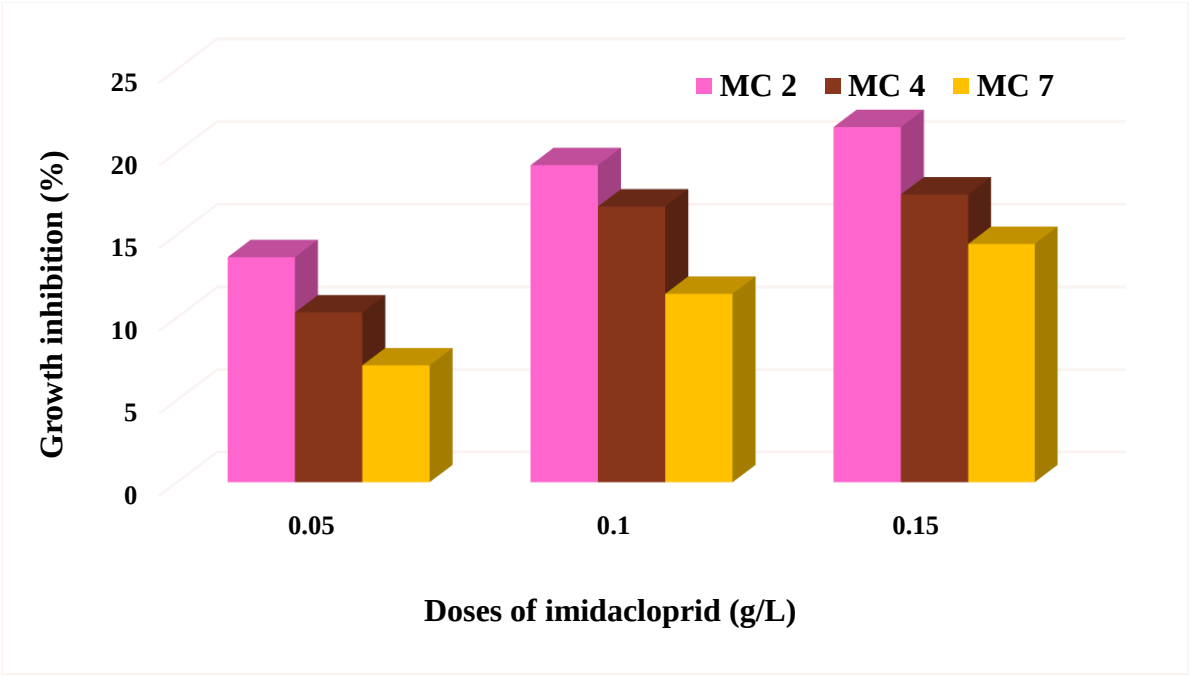


Fig 10. Effect of imidacloprid on the growth of *Metarhizium anisopliae* isolates

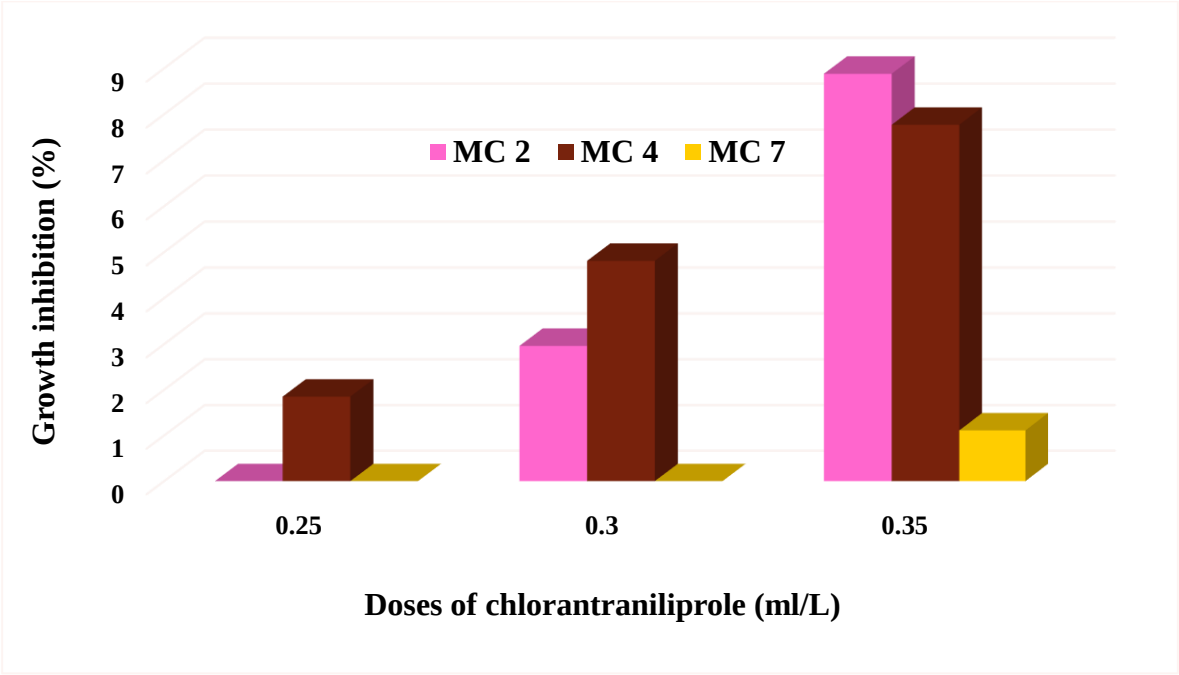


Fig 11. Effect of chlorantraniliprole on the growth of *Metarhizium anisopliae* isolates

Screening of isolates for fungicide compatibility were also carried out. Less than eight per cent growth inhibition was observed for all isolates at different doses of the fungicide, copper oxychloride (COC) proving its compatibility with *M. anisopliae*. Among the three isolates tested, MC 2 recorded least growth inhibition of 1.11 to 3.70 per cent at different doses of copper oxychloride (Fig 12). However, higher sporulation was exhibited by the isolate MC 7, with medium sporulation even at the highest dose of COC (0.30 g/L). According to Mochi *et al*. (2005), CO_2 production by *M. anisopliae* was suppressed in soil for 4-6 days when co- applied with fungicides (COC, tebuconazole *etc*), but after that there is no significant difference between the respiratory activity of *M. anisopliae* in fungicide treated and untreated soil. The tested acaricides, herbicides and insecticides had only less impact on respiratory activity of fungi and hence suggested for the combined application with fungi. The insecticides used in the present study were more compatible with *M. anisopliae* isolates than the fungicides used and were in line with the reports of Mochi *et al*. (2006) who studied the effects of insecticides and fungicides in the growth of *M. anisopliae*. Most of the fungicides were incompatible with the entomopathogens while there was a greater compatibility between insecticides and *M. anisopliae*.

Growth of all isolates was considerably inhibited even at the lowest dose of carbendazim (0.50 g/L). Total growth inhibition was observed in all isolates at recommended dose of 1 g/L and above. At 0.50 g/L, the inhibition in the growth of all isolates were ranged between 58.51 and 86.33 per cent. MC 2 and MC 7 registered medium and high sporulation respectively at the lowest dose of fungicide. No sporulation was observed at higher doses (Fig 13).

All isolates of *M. anisopliae* resulted in 100 per cent growth inhibition at all doses of hexaconazole (1.50, 2, 2.50 ml/L) depicting that the isolates obtained in the present study were incompatible to fungicide hexaconazole.

In the present study, all the isolates showed a drastic decrease in growth and sporulation in the PDA amended with fungicides when compared to insecticides. These results were in line with the reports of Mochi *et al*. (2005 and 2006) who studied the effects of insecticides and fungicides in the growth of *M. anisopliae* and concluded that most of the fungicides were incompatible with the entomopathogens while there was greater compatibility between insecticides and *M. anisopliae*. Moreover, laboratory bioassays alone doesn't determine the effective compatibility of entomopathogens with pesticides hence additional field or greenhouse studies are required to confirm the compatibility or incompatibility of pesticides with biocontrol agents before they recommend in crop management strategies.

5.3. BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *M. anisopliae*

Microorganisms exhibits various defensive mechanisms to cope up with extreme environmental conditions, which involve enzymatic activity as well as production of biomolecules. In the present study, influence of thermal and water stresses on the activity of catalase, peroxidase and esterase as well as variations in the quantity of trehalose produced were assessed.

5.3.1. Catalase, Peroxidase and Esterase Activity of Stress Tolerant Isolates of *Metarhizium anisopliae*

Catalases and peroxidases are antioxidant enzymes involved in the detoxification of H_2O_2 accumulated as part of physiological processes and abiotic stresses in fungi. These defensive enzymes enable the cells to cope up with extreme environmental conditions. Esterase is an essential intracellular enzyme needed for infection process. According to Hisada *et al*. (2005), catalases are essential in reducing toxic effects of oxidative stress that are induced by hydrogen peroxide.

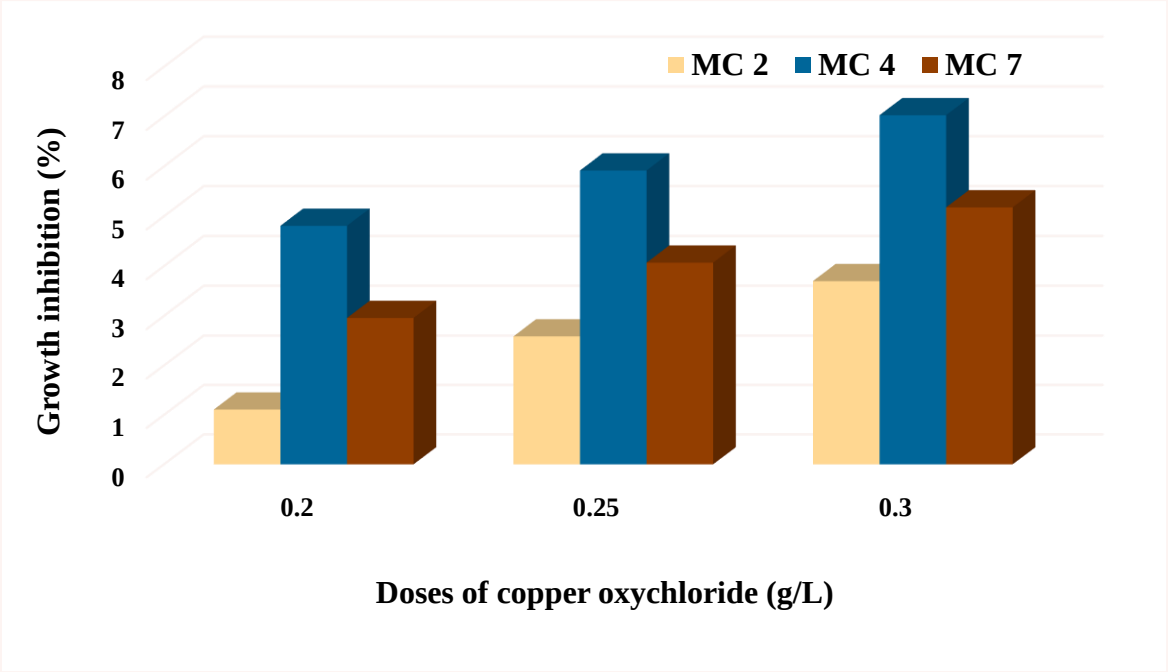


Fig 12. Effect of copper oxychloride on the growth of *Metarhizium anisopliae* isolates

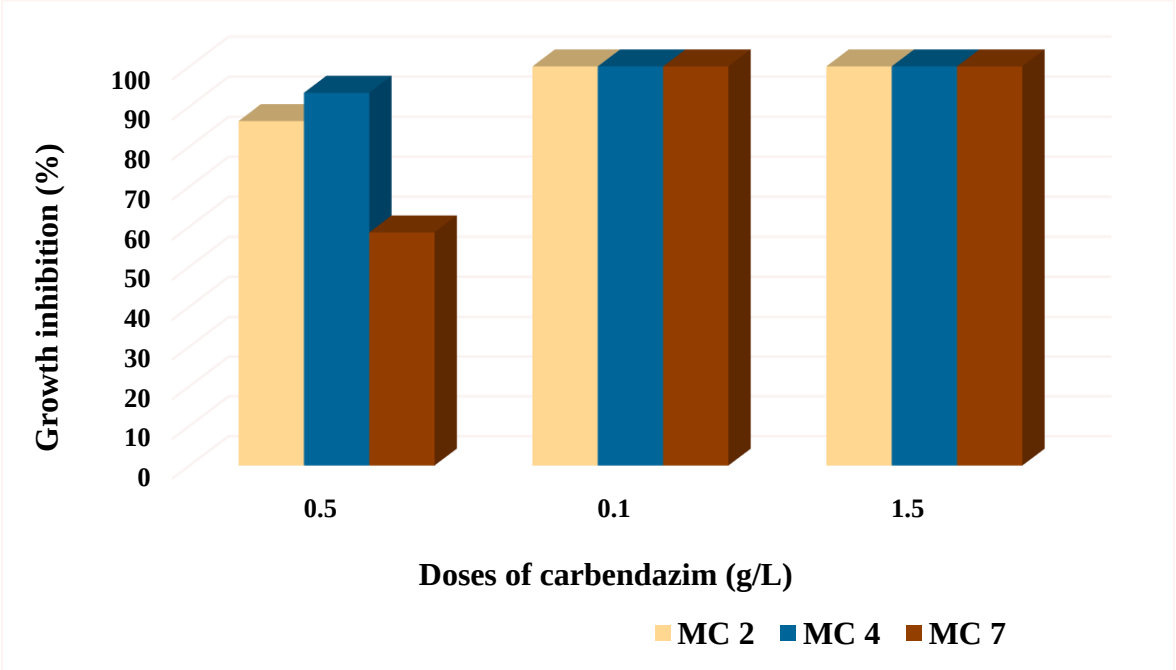


Fig 13. Effect of carbendazim on the growth of *Metarhizium anisopliae* isolates

Significant reduction in esterase activity, from 0.036 to 0.012 µmol/min/mg protein was observed in case of MC 7 at higher temperatures (Fig 14). Likewise, MC 2 demonstrated a reduction in esterase activity from 0.050 to 0.015 µmol/min/mg protein when exposed to increased PEG concentrations (Fig 16).

In the present study, the thermotolerant isolate MC 7 recorded elevation of both catalase and peroxidase at higher temperatures and similar results were also obtained for the drought tolerant isolate MC 2 which recorded higher enzyme activities at higher concentrations of PEG. Isolate MC 7 showed significantly highest catalase activity of 0.140 EU/min/mg protein and peroxidase activity of 0.058 EU/min/tissue weight at 37ºC than at lower temperatures (Fig 14). Similarly, MC 2 also recorded highest catalase activity of 0.294 EU/min/mg protein and peroxidase activity of 0.030 EU/min/tissue weight at 35 per cent concentration of PEG. However, both MC 7 and MC 2 exhibited an inverse relationship to abiotic stresses in terms of esterase activity (Fig 16).

The above observations were in congruence with the findings of Sujatha and Padmaja (2014) where they had noticed a considerable increase in antioxidant enzymes, catalase and peroxidase and a slight loss in intracellular enzyme, esterase when *M. anisopliae* exposed from 25ºC to 37ºC. Angelova *et al*. (2005) reported that heat shock and oxidative stress stimulated higher induction of peroxidase in *Neurospora crassa*. Similarly Machwe *et al*. (2002) also noticed a 6-fold increase of peroxidase activity in *N. crassa* cells under heat shock. Bilinski (1988) reported an induction of catalase in *Streptomyces cerevisiae* at temperature of 22 to 37ºC. According to Padmini and Padmaja (2014), catalase and peroxidase activity in *M. anisopliae* increased as temperature increased from 25 to 33ºC.

5.3.2. Trehalose Content in the Stress Tolerant Isolates of *Metarhizium anisopliae*

Trehalose hydrolysis is important in various physiological processes like spore germination, restoration of resting cells and prevention of damage to cells by abiotic stresses. Singer and Lindquist (1998) found that trehalose helped in stabilizing native proteins and reduced aggregation of denatured proteins under high temperature.

Present study revealed that trehalose content of thermotolerant isolate MC 7 had increased from 0.760 to 1.380 mg/min/g mycelia with increase in temperature from 27ºC to 37ºC (Fig 15). Similarly, significantly higher levels of trehalose (1.580 mg/min/g mycelia) was observed in MC 2 at highest concentration of PEG when compared to the quantity of trehalose in control (0.668 mg/min/g mycelia) [Fig 17].

Above findings were in agreement with the reports of Kandror *et al*. (2004) as they had reported that fungal cells were known to accumulate trehalose upto 30 per cent in response to various stresses like heat shock, water stress and nutritive stress. Similarly, Eleutherio *et al*. (1993) and Hottiger *et al*. (1987) noticed that the cells accumulated trehalose in response to increasing temperature, cold shock, dehydration, osmotic stress *etc*. Thevelein (1984) reported that high concentration of trehalose and polyols in conidia could impart stress tolerance in many entomopathogenic fungi. Jin *et al*. (2015) suggested that virulence and *in vivo* growth of *M. acridum* was contributed by the sugar trehalose. Hallsworth and Magan (1994 and 1996), evaluated the amount of polyols and trehalose accumulated by entomopathogens by varying temperatures and pH. Conidia of *M. anisopliae* registered greater amounts of trehalose between temperatures of 20 and 30ºC and pH values between 4 and 8.

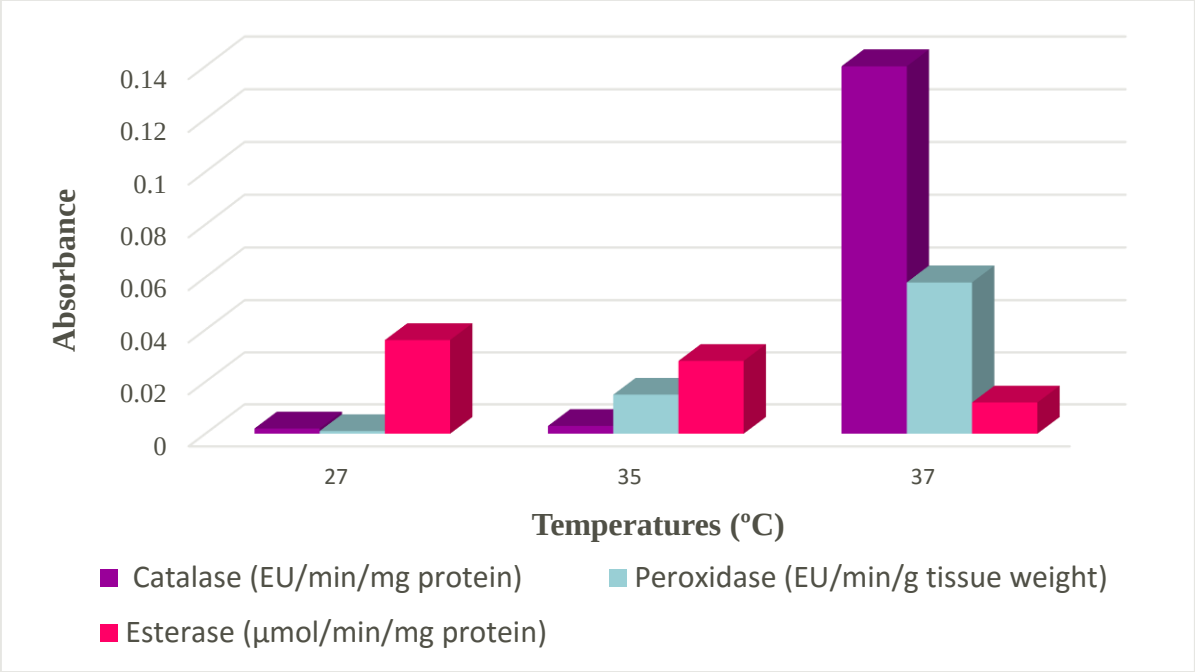


Fig 14. Enzyme activity of thermotolerant isolate (MC 7) of *Metarhizium anisopliae*

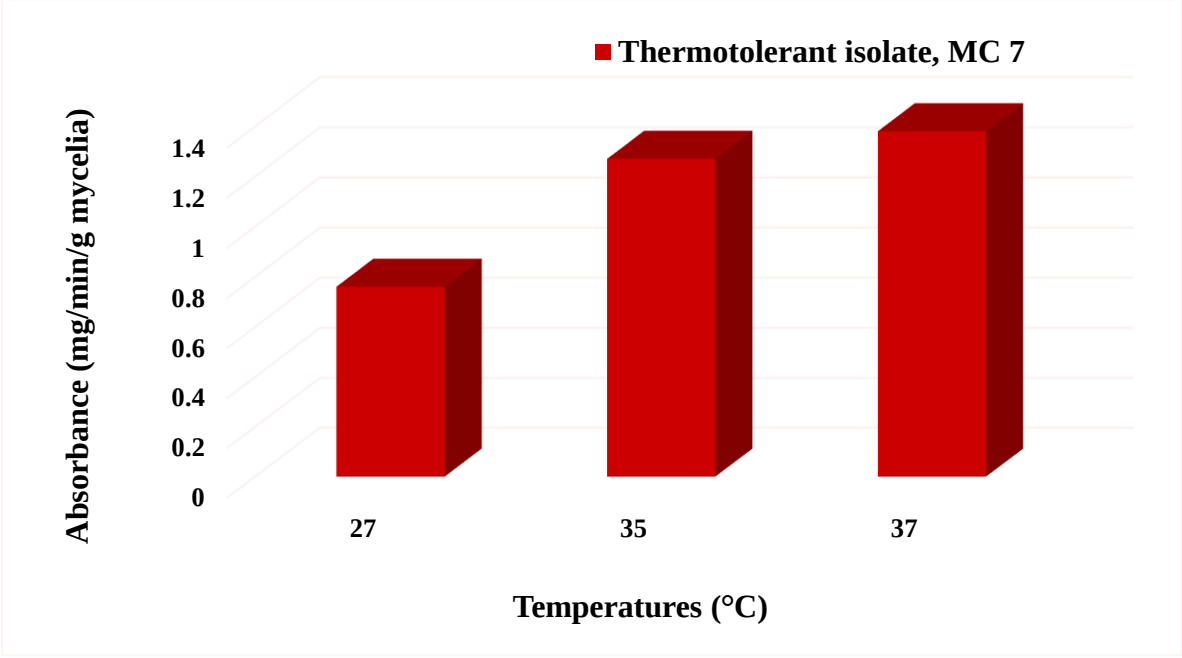


Fig 15. Quantity of trehalose in the thermotolerant isolate (MC 7) of *Metarhizium anisopliae*

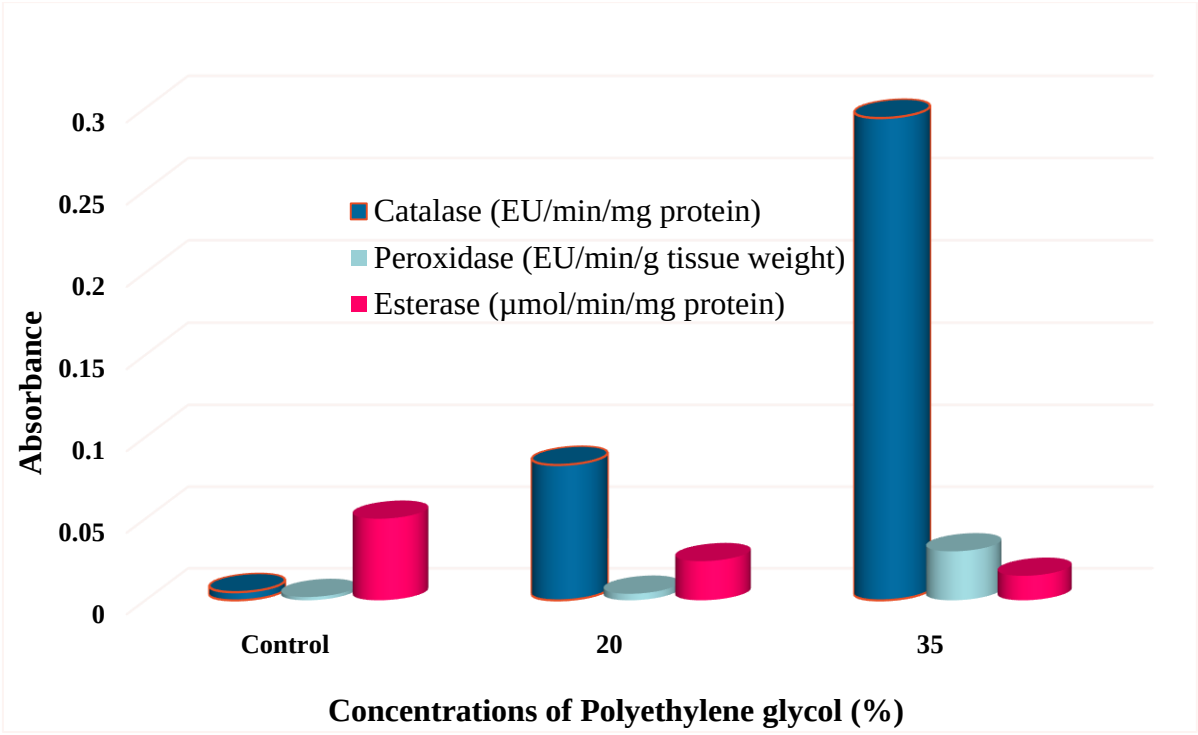


Fig 16. Enzyme activity of drought tolerant isolate (MC 2) of *Metarhizium anisopliae*

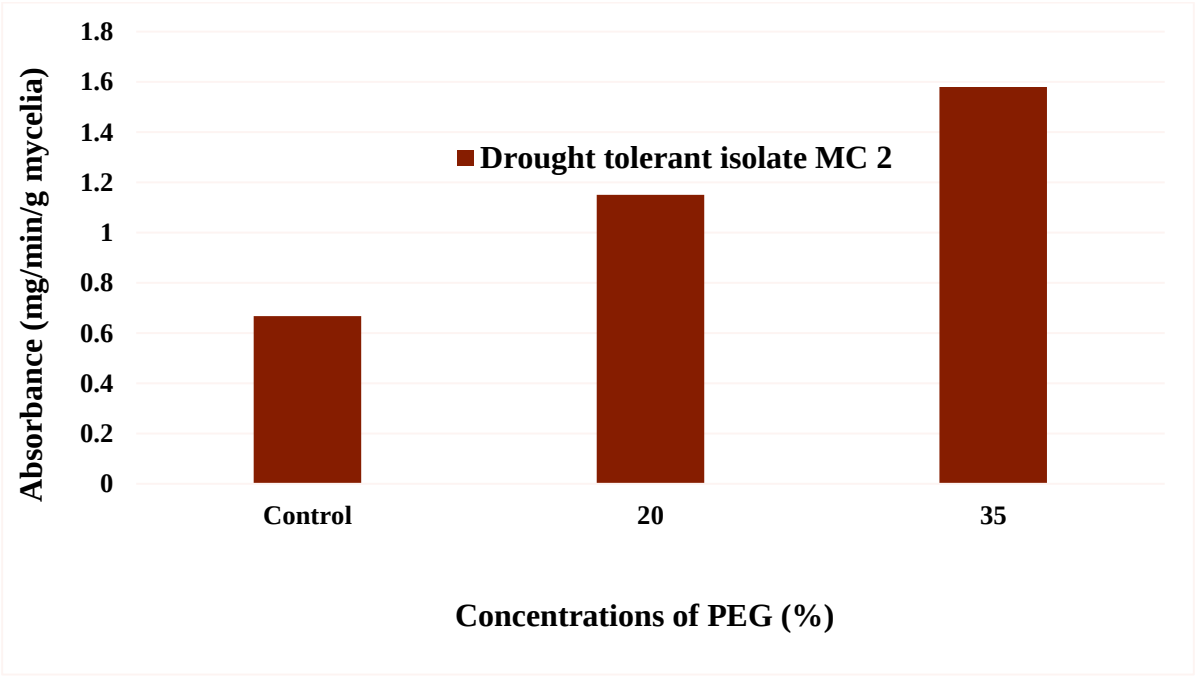


Fig 17. Quantity of trehalose in the drought tolerance isolate (MC 2) of *Metarhizium anisopliae*

5.4. *IN VITRO* EVALUTION OF STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* AGAINST *Tribolium castaneum*

Efficacy of stress tolerant isolates of *M. anisopliae* against the storage pest *Tribolium castaneum* was evaluated. In adults, all isolates caused 10 to 20 per cent mortality after six days of treatment at 10^8 to 10^9 spores/ml and no mortality was found in the isolate MC 4 at concentrations of 10^5 to 10^7 spores/ml. At lowest dose of 10^5 spores/ml, isolate MC 7 recorded significantly lowest LT_{50} and LT_{90} of 15.12 and 23.32 days than other isolates. Highest LT_{50} and LT_{90} of 16.40 and 26.58 days were recorded by the isolate MC 4.

Isolate MC 2 recorded 86.66 per cent mortality at highest concentration of 10^9 spores/ml fifteen days after treatment with a LT_{50} and LT_{90} of 9.43 and 16.01 days while isolate MC 7 showed 83.33 per cent mortality, recorded LT_{50} and LT_{90} values of 10.07 and 16.42 days. This was on par with the positive check, which caused a mortality of 86.66 per cent with a LT_{50} and LT_{90} of 9.06 and 15.45 days. But in case of the isolate MC 4, only 70 per cent mortality was recorded with highest LT_{50} and LT_{90} (Fig 18).The above results were found in congruence with the findings of Komaki *et al*. (2017). They noticed variation in the mortality rate of red flour beetles caused by the entomopathogenic fungi was between 34.6 to 100 per cent at ten days after treatment.

Padin and Vasicek (1997) elucidated the pathogenicity of *B. bassiana* on red flour beetle and observed that conidia of 0.5 g per 20 insects recorded mortality of 87 per cent within 21 days of exposure.

In case of mortality of grubs caused by the isolates, 13.33 to 23.33 per cent mortality was caused by the isolate MC 2 after three days of treatment at doses of 10^7 to 10^9 spores/ml, which was on par with positive control, causing similar mortality rates at the same concentrations. It was then followed by the isolate MC 7 which recorded a mortality of 10 to 20 per cent in grubs. Lowest mortality after three

days of treatment was recorded by the isolate MC 4 with mortality ranging between 10 and 16.66 per cent when applied at 10^8 and 10^9 spores/ml.

At lower concentration of 10^5 spores/ml, lowest LT_{50} and LT_{90} were recorded in the isolate MC 2 with a value of 6.09 and 9.27 days followed by the isolate MC 7 with a LT_{50} and LT_{90} of 6.85 and 10.76 days. Similarly, LT_{50} and LT_{90} values of 7.04 and 9.54 days were also observed in positive control. Highest LT_{50} value of 9.54 days was noticed in the isolate MC 4. At 10^9 spores/ml, isolate MC 2 showed the least LT_{50} and LT_{90} of 4.14 and 6.58 days followed by the isolate MC 7 with values of 4.62 and 7.67 days. These isolates were found on par with the positive control exhibiting LT_{50} and LT_{90} of values 4.13 and 6.38 days (Fig 19). Rachappa *et al*. (2009) evaluated the bioefficacy of different isolates of *M. anisopliae* against three test insects like *Helicoverpa armigera, Plutella xylostella* and *Oryctes rhinoceros*. Isolate Ma2 was found to be more virulent with less LC_{50} values of 1.77 to 1.98 × 10^6 spores/ml against *H. armigera* and *P. xylostella*. Lethal time taken (LT_{50}) was very low in the isolate Ma2 with value of 126.15 h. Isolate Ma1 was the best isolate showing more virulence to *O. rhinoceros*. Sun *et al*. (2016) noticed 60 per cent mortality affected by *M. anisopliae* in grubs of red palm weevil by ten days of post inoculation at lowest dose of 10^6 spores/ml but when the dose applied was increased to 10^8 spores/ml, mortality was increased to 100 per cent. According to Reddy *et al*. (2017), *M. anisopliae* caused 98 per cent mortality in the larva of *H. armigera* by seventh day at the dose of 10^8 spores/ml and 100 per cent mortality was assured by eighth day of treatment.

Present study revealed that, among the three *M. anisopliae* isolates, MC 7 has the potential to withstand high temperatures and acidity levels and hence can be considered as a thermo tolerant and acidity tolerant. MC 2 which could survive at higher salinity levels as well as drought conditions can be selected as a tolerant isolate to both salinity and drought. All the isolates were compatible to the insecticides spinosad, imidacloprid and chlorantraniliprole as well as fungicide,

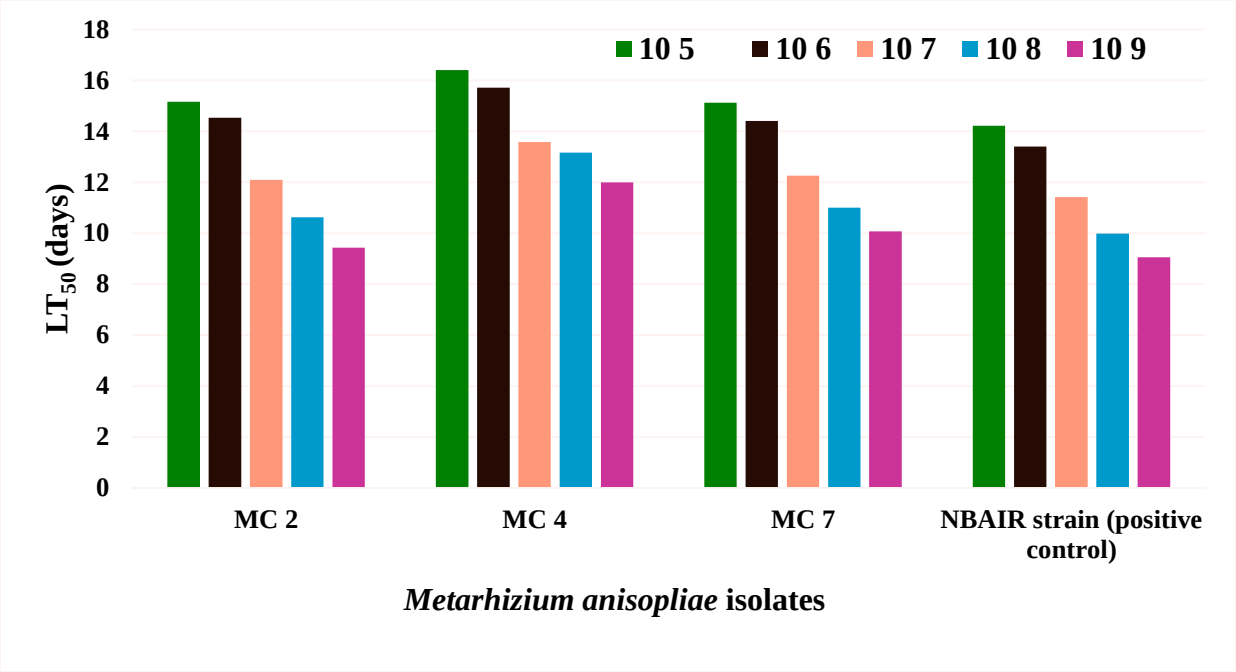


Fig 18. LT_{50} caused by *Metarhizium anisopliae* isolates on adults of *Tribolium castaneum*

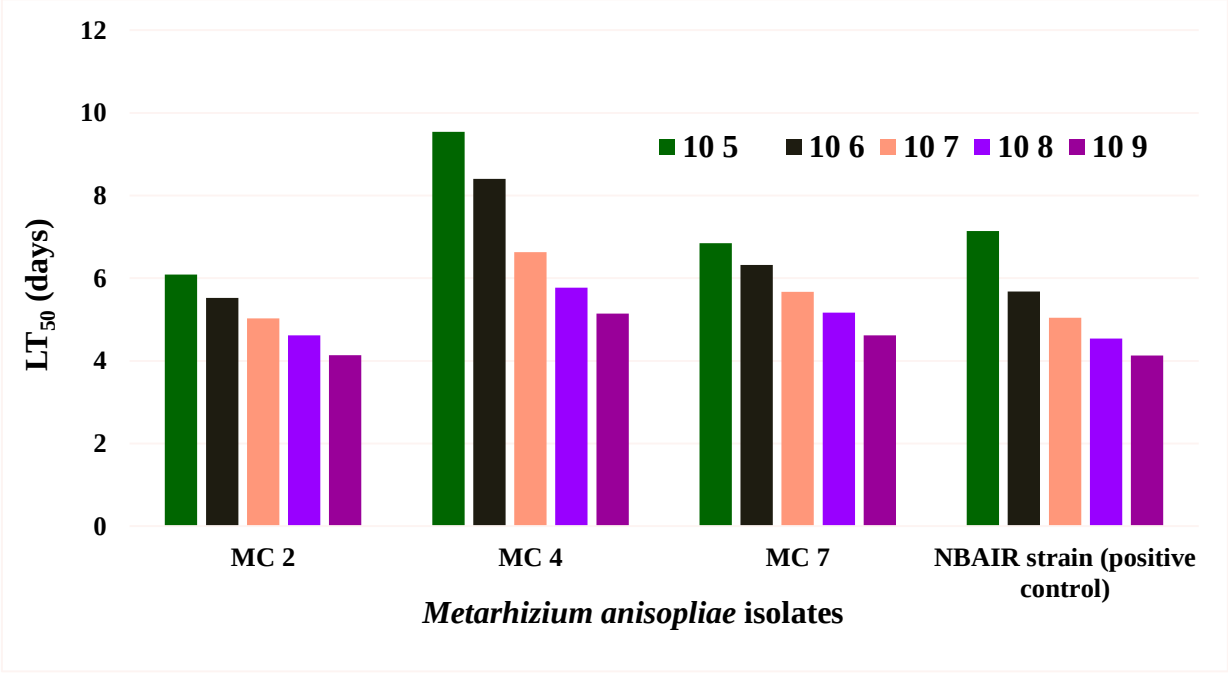


Fig 19. LT_{50} caused by *Metarhizium anisopliae* isolates on grubs of *Tribolium castaneum*

copper oxychloride at all doses tested and hence combined application of these pesticides with *M. anisopliae* isolates MC 2, MC 4 and MC 7 is suggested. The defensive enzymes *viz*., catalase and peroxidase were elevated during stress conditions and levels of these enzymes can be used as biochemical markers for screening temperature and drought tolerance in EPF. Bio efficacy studies also proved that the isolates MC 2 and MC 7 had effectively managed populations of red flour beetle at higher spore concentrations. Considering all these factors, the multiple stress tolerant isolates, MC 7 and MC 2 can be selected as an ideal candidates for integrated pest management programmes.

Summary

6. SUMMARY

Entomopathogenic fungi (EPF) are widely known biocontrol agents of pests and application of EPF as mycoinsecticide is of great significance due to environmental and food safety concerns. *Metarhizium* is one among the EPF, a typical soil-borne fungus, effective against a wide range of insect species. However, field efficacy of *Metarhizium* formulations is uncertain under various environmental stresses. Hence, the present research work entitled "Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorkin" was conducted in the Department of Agricultural Entomology, College of Hroticulture, Vellanikkara, Thrissur during 2017-2019. It comprised of identifying different abiotic stress tolerant isolates of *M. anisopliae* from Kerala soil and to evaluate its tolerance to various stresses and also its bioefficacy against test insect, *Tribolium castaneum*.

The striking findings of the study are summarized below.

- Soil samples were collected through purposive survey from areas with abiotic stresses in Kerala *viz*., Thrissur, Palakkad, Ernakulam, Idukki and Alappuzha.
- Physico-chemical properties of the collected soil samples such as soil texture, temperature, moisture content, pH and electrical conductivity were analysed. Among the 40 soil samples, highest soil temperature (46ºC) and lowest soil moisture content (1.10 %) were recorded from sample Kallampatti 2 and Kallampatti 1 of Palakkad district. Sample Moncompu 1 of Alappuzha district recorded lowest soil pH (3.40) and highest EC (6.68 dSm^{-1}).
- Through isolation and enumeration for *Metarhizium anisopliae* by Veen's selective medium as well as bait trap method, five soil samples from Alappuzha district (Moncompu 2, 3, 4, 7 and 8) alone showed population and mycosed larva due to *Metarhizium*. Highest population was obtained from the soil sample

collected from the location Moncompu 7 (19.66×10^{-3}cfu/ml and 7.33×10^{-4}cfu/ml).

- A total of three isolates of *M. anisopliae* were identified by cultural, morphological and molecular characterization. The isolates were abbreviated and serially numbered based on the name and number of location such as MC 2, MC 4 and MC 7 representing isolates from Alappuzha district.
- Evaluation of isolates for acidity tolerance at different pH levels (5.5, 4.5, 3.5, 3 and 2.5) revealed that, isolate MC 7 produced highest mycelial biomass (1.15 g) at lowest pH of 2.5 and was able to sporulate moderately at pH 3.Thus, MC 7 was selected as acidity tolerant isolate.
- At highest concentration of 1.5 M NaCl, used in the study, isolate MC 2 exhibited good growth with only 58.84 per cent inhibition and sparse sporulation was also observed at 1 M NaCl concentration, claiming as saline tolerant isolate.
- Isolates of *M. anisopliae* were grown at different temperatures of 25, 30, 35, 37 and 40°C in order to screen better isolate which can survive well at higher temperatures. Among the isolates, MC 7 recorded highest mycelial biomass (1.28 g) with sparse sporulation at highest temperature, 37°C, considered as a thermotolerant isolate in this study.
- Screening of isolates for drought tolerance by amending different concentrations of PEG in PDA revealed that, isolate MC 2 recorded highest mycelial weight (0.93 g) at highest concentration of PEG, (35 per cent) and also showed sparse sporulation at 30 per cent PEG. Hence, MC 2 was selected as drought tolerant.
- Compatibility study of isolates to popular insecticides and fungicides at different doses (lower dose, recommended dose and higher dose) noticed that, all the isolates were found compatible to the insecticides spinosad, imidacloprid and chlorantraniliprole as well as fungicide copper oxychloride. Among the isolates, MC 2 and MC 7 were more compatible to these chemicals.

- Biochemical characterization of stress tolerant isolates (MC 2 and MC 7) were carried out in order to study the variation in the enzyme activity and quantity of trehalose with respect to stress. Highest esterase activity was obtained from the isolate MC 7 when grown at room temperature of 27°C (0.036 µmol/min/mg protein) and MC 2 grown in PDB without PEG (0.050µmol/min/mg protein) when compared to the esterase activity of the isolates grown at 37°C and 35 per cent PEG.
- Isolate MC 7 when grown at highest temperature of 37°C and MC 2 at highest concentration of 35 per cent PEG recorded highest peroxidase activities (0.058 and 0.030 EU/min/g tissue weight) when compared to control with a values of 0.001 and 0.002 EU/min/g tissue weight).
- Highest catalase activity was recorded by the isolate MC 7 when grown at highest temperature of 37°C and at highest concentration of PEG 35 per cent in PDB (0.140 and 0.294 EU/min/mg protein). Lowest activities of catalase was recorded in the control with a values of 0.002 and 0.005 EU/min/mg/mg protein.
- Quantity of trehalose in the mycelia was higher at highest temperature of 37°C (MC 7) and at highest concentration of 35 per cent PEG (MC 2) with values 1.380 and 1.580 mg/min/g mycelia when compared to control with values of 0.760 and 0.668 mg/min/g mycelia.
- Evaluation of stress tolerant isolates against a storage pest, *Tribolium castaneum* concluded that all the stress tolerant isolates were found to be on par with the positive control. In adults, isolate MC 2 at 10^9 spores/ml recorded LT_{50} and LT_{90} values of 9.43 and 16.01 days while isolate MC 7 recorded LT_{50} and LT_{90} values of 10.07 and 16.42 days. Without any significant difference, positive check registered LT_{50} and LT_{90} values of 9.06 and 15.45 days.
- In case of grubs, isolate MC 2 showed least LT_{50} and LT_{90} values of 4.14 and 6.58 days at 10^9 spores/ml followed by the isolate MC 7 with a values of 4.62 and 7.67

days. The records were found on par with the positive control exhibiting LT_{50} and LT_{90} of values 4.13 and 6.38 days.

- Biocontrol efficacy test of stress tolerant isolates against *Tribolium castaneum* proved that the stress tolerant isolates MC 2 and MC 7 had good potential in managing the test insect which was comparable to that of positive check.

References

7. REFERENCES

Abbot, W. S. 1925. A method for computing the effectiveness of insecticides. *J. Econ. Entomol*. 18: 265-267.

Akbar, S., Freed, S., Hameed, A., Gul, H. T., Akmal, M., Malik, M. N., Naeem, M., and Khan, M. B. 2012. Compatibility of *Metarhizium anisopliae* with different insecticides and fungicides. *Afr. J. Microbiol. Res.* 6(17): 3956-3962.

Amalraj, L. D. E., Kumar, P. G., Desai, S., and Ahmed, H. M. 2010. *In vitro* characterization of *Trichoderma viride* for abiotic stress tolerance and field evaluation against root rot disease in *Vigna mungo* L. *J. Biofertil. Biopestici*. 2(3): 1-5.

Angelova, M. B., Pasgiva, S. B., Spasova, B. K., Vassilev, S. V., and Slokoska, L. S. 2005. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. *Mycol. Res*. 109(2): 150-158.

Asensio, L., Carbonell, T., Lopez-Jimenez, J. A., and Lopez-Llorca, L. V. 2003. Entomopathogenic fungi in soils from Alicante province. *Spanish J. Agric. Res*. 1(3): 37-45.

Ayala-Zermeno, M. A., Berlanga-Padilla, A. G. A. M., Andrade-Michel, G. Y., Rodriguez-Rodriguez, J. C., Arredondo-Bernal, H. C., and Montezinos-Matias, R. 2017. Viability, purity and genetic stability of entomopathogenic fungi species using different preservation methods. *Fungal Biol.* 121: 920-928.

Bastin, B., Beena, V. I., Sreelatha, A. K., and Dey, P. 2014. *GPS and GIS Based Modelmsoil Fertility Maps for Precise Fertilizer Recommendations to the Farmers of Kerala.* Kerala Agricultural University, Thrissur, Kerala, 161p.

Batta, Y. A. and Safieh, D. I. A. 2005. A study of treatment effect with *Metarhizium anisopliae* and four types of dusts on wheat grain infestation with red flour beetles (*Tribolium castaneum* Herbst, Coleoptera: Tenebrionidae). *J. Nat. Stud. Eng*. 13(1): 11-22.

Baverstock, J., Roy, H. E., and Pell, J. K. 2010. Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors. *Biocontrol* 55: 89-102.

Bhatia, S. K. and Pradhan, S. 1968. Studies on resistance to insecticides in *Tribolium castaneum* (Herbst) I- Selection of a strain resistant to p, p'-DDT and its biological characteristics. *Indian J. Entomol*. 30: 13-32.

Beevi, S. N., Paul, A., George, T., Mathew, T. B., Kumar, N. P., Xavier, G., Kumar, G. T. P., Rajith, R., Ravi, K. P., and Kumar, S.V. 2014. Pesticide residues in soils under cardamom cultivation in Kerala, India. *Pesticide Res. J.* 26(1): 35-41.

Behie, S. W., Zelisko, P. M., and Bidochka, M. J. 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Sci*ence 336: 1576–1577.

Bell, J. V. and Hamalle, R. J. 1974. Viability and pathogenicity of entomogenous fungi after prolonged storage on silica gel at -20°C. *Can. J. Microbiol.* 20(5): 639-642.

Bidochka, M. J., Kasperski, J. E., and Wild, G. A. M. 1998. Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near northern habitats. *Can. J. Bot.* 76: 1198-1204.

Bilinski, T. 1988. Mechanisms of oxygen toxicity as revealed by studies of yeast mutants with changed response to oxidative stress. *Oxy-Radicals Mol. Biol. Pathol*. 109-123.

Borisade, O. A. and Magan, N. 2014. Growth and sporulation of entomopathogenic *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria farinosa* and *Isaria fumosorosea* strains in relation to water activity and temperature interactions. *Biocontrol Sci. Technol.* 24(9): 999-1011.

Bridge, P. D., Williams, M. A. J., Prior, C., and Paterson, R. R. M. 1993. Morphological, biochemical and molecular characteristics of *Metarhizium anisopliae* and *M. flavoviride*. *Microbiology* 139(6): 1163-1169.

Brunner-Mendoza, C., Reyes-Montes, M. D. R., Moonjely, S., Bidochka, M. J., and Toriello, C. 2019. A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. *Biocontrol Sci. Technol*. 29(1): 83-102.

Butt, T. M., Jackson, C. W., and Magan, N. 2001. *Fungi as Biocontrol Agents, Progress, Problems and Potential*. CAB International publishing, UK, 390p.

Cavalcanti, M. A. D. Q. 1991. Viability of Basidiomycotina cultures preserved in mineral oil. *Rev. Latinoamerican Microbiol.* 32: 265-268.

Chen, W. Q. J. and Zhu, T. 2004. Networks of transcription factors with roles in environmental stress response. *Trends Plant Sci*. 9: 591-596.

Chen, Z. H., Xu, L., Yang, F. L., Ji, G. H., Yang, J., and Wang, J. Y. 2014. Efficacy of *Metarhizium anisopliae* isolate MAX-2 from Shangri-la, China under desiccation stress. *Microbiology* 14(1): 4.

Dimbi, S., Maniania, N. K., Lux, S. A., and Mueke, J. M. 2004. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *Biocontrol* 49(1): 83-94.

Domsch, K. H., Gams, W., and Anderson, T. H. 1980. *Compendium of Soil Fungi*. Academic Press, London, United Kingdom, 860p.

Ekesi, S., Maniania, N. K., and Lux, S. A. 2003. Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. *J. Invertebrate Pathol.* 83(2): 157-167.

Ekesi, S., Maniania, N. K., Mohamed, S. A., and Lux, S. A. 2005. Effect of soil application of different formulations of *Metarhizium anisopliae* on African tephritid fruit flies and their associated endoparasitoids. *Biol. Control* 35: 83-91.

Eleutherio, E. C. A., Araujo, P. S., and Panek, A. D. 1993. Protective role of trehalose during heat stress in *Saccharomyces cerevisiae*. *Cryobiology* 30: 591-596.

Faria, M., Hajek, A. E., and Wraight, S. P. 2009. Imbibitional damage in conidia of the entomopathogenic fungi *Beauveria bassiana*, *Metarhizium acridum* and *Metarhizium anisopliae*. *Biol. Control* 51: 346-354.

Faria, M., Lopes, R. B., Souza, D. A., and Wraight, S. P. 2015. Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi. *J. Invertebrate Pathol*. 125:68-72.

Fernandes, E. K. K., Keyser, C. A., Rangel, D. E. N., Foster, R.N., and Roberts, D. W. 2010. CTC medium: a novel dodine-free selective medium for isolating entomopathogenic fungi, especially *Metarhizium acridum*, from soil. *Biol. Control* 54(3): 197-205.

Filho, A. B., Almeida, J. E. M., and Lamas, C. 2001. Effect of thiamethoxam on entomopathogenic microorganisms. *Neotropical Entomol.* 30(3): 437-447.

Finney, D. J. 1971. *Probit Analysis.* (3rd Ed.). University Press, Cambridge, U.K. 333p.

Gal-Hemed, I., Atanasova, L., Komon-Zelazowska, M., and Yarden, O. 2011. Marine isolates of *Trichoderma* as potential halotolerant agents of biological control for arid-zone agriculture. *Appl. Environ. Microbiol.* 77(15): 5100-5109.

Ghayedi, S. and Abdollahi, M. 2013. Biocontrol potential of *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae), isolated from suppressive soils of the Boyer-ahmad region, Iran, against J2S of *Heterodera avenae*. *J. Plant Prot. Res*. 53: 165-171.

Glare, T. R., Milner, R. J., and Beaton, C. D. 1996. Variation in *Metarhizium*, a genus of fungal pathogens attacking Orthoptera: Is phialide morphology a useful taxonomic criterion?. *J. Orthoptera Res*. 5: 19-27.

Gomez, K. A. and Gomez, A. A. 1984. *Statistical Procedures for Agricultural Research*. John Wiley and Sons, Australia, 639p.

Grover, R. K. and Moore, J. D. 1962. Toximetric studies of fungicides against brown rot organism *Sclerotina fruticola*. *Phytopathol*ogy 52: 876-880.

Hallsworth, J. E. and Magan, N. 1994. Effect of KCl concentration on accumulation of acyclic sugar alcohols and trehalose in conidia of 3 entomopathogenic fungi. *Lett. Appl. Microbiol.* 18: 8-11.

Hallsworth, J. E. and Magan, N. 1995. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology* 141: 1109-1115.

Hallsworth, J. E. and Magan, N. 1996. Culture age, temperature and pH affect the polyol and trehalose contents of fungal propagules. *Appl. Environ. Microbiol.* 62(7): 2435-2442.

Hallsworth, J. E. and Magan, N. 1999. Water and temperature relations of growth of the entomogenous fungi *Beauveria bassiana, Metarhizium anisopliae* and *Paecilomyces farinosus*. *J. Invertebrate Pathol*. 74: 261-266.

Harris, J. L. 1986. Modified method for fungal slide culture. *J. Clin. Microbiol*. 24: 460-461.

Hasan, W. A., Assaf, L. H., and Abdullah, S. K. 2012. Occurrence of entomopathogenic and other opportunistic fungi in soil collected from insect hibernation sites and evaluation of their entomopathogenic potential. *Bull. Iraq Nat. Hist. Museum* 12(1): 19-27.

Hernandez, C. E. M., Guerrero, I. E. P., Hernandez, G. A. G., Solis, E. S., and Guzman, J. C. T. 2010. Catalase overexpression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*. *Appl. Microbiol. Biotechnol.* 87(3): 1033-1044.

Hisada, H., Hata, Y., Kawato, A., Abe, Y., and Akita, O. 2005. Cloning and expression analysis of two catalase genes from *Aspergillus oryzae*. *J. Biosci. Bioeng*. 99: 562-568.

Hottiger, T., Schmutz, P., and Wiemken, A. 1987. Heat-induced accumulation of futile cycling of trehalose in *Saccharomyces cerevisiae*. *J. Bacteriol*. 169: 5518-5522.

Hu, G. and Leger, R. J. S. 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol.* 68(12): 6383-6387.

Humber, R. A. 1997. Fungi: preservation of cultures. In *Manual of techniques in insect pathology*. Academic Press. pp. 269-279.

Im, D. J., Lee, M. H., Aguda, R. M., and Rombach, M. C. 1988. Effect of pH and nutrients on the growth and sporulation of four entomogenous hypomycetes fungi (Deuteromycotina). *Korean J. Appl. Entomol*. 27(1): 41-46.

Imoulan, A., Alaoui, A., and Meziane, A. E. 2011. Natural occurrence of soil-borne entomopathogenic fungi in the Moroccan Endemic forest of *Argania spinosa* and their pathogenicity to *Ceratitis capitata*. *World J. Microbiol. Biotechnol*. 27: 2619-2628.

Indira, B. V. N. and Covilakom, M. T. K. 2013. Characterization of acidity in acid sulphate soils of Kerala. *J. Life Sci.* 7(8): 907.

Jackson, M. L. 1958. *Soil Chemical Analysis: Advanced Course*. UW-Madison Libraries Parallel Press, London, 925p.

Jacob, S., Resmi, G., and Mathew, P. K. 2015. Environmental pollution due to pesticide application in cardamom hills of Idukki District, Kerala, India. *Int. J. Basic Appl. Sci*. 1: 27-34.

Jin, K., Peng, G., Liu, Y., and Xia, Y. 2015. The acid trehalase, ATM1, contributes to the *in vivo* growth and virulence of the entomopathogenic fungus, *Metarhizium acridum*. *Fungal Genet. Biol.* 77: 61-67.

Kamath, M. N., Patel, M. K., and Dhande, G. W. 1952. Occurrence of green muscardine fungus on *Pyrilla* sp. *Curr. Sci*. 21: 317.

Kamp, A. M. and Bidochka, M. J. 2002. Conidium production by insect pathogenic fungi on commercially available agars. *Lett. Appl. Microbiol*. 35(1): 74-77.

Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D., and Goldberg, A. L. 2004. Yeast adapt to near-freezing temperatures by STRE/Msn2, 4-dependent induction of trehalose synthesis and certain molecular chaperones. *Mol. Cell* 13: 771-781.

Kavitha, C. and Sujatha, M. P. 2015. Evaluation of soil fertility status in various agro ecosystems of Thrissur District, Kerala, India. *Int. J. Agric. Crop Sci*. 8(3): 328.

Keller, S., Kessler, P., and Schweizer, C. 2003. Distribution of insect pathogenic soil fungi in Switzerland with special reference to *Beauveria brongniartii* and *Metarhizium anisopliae*. *Biocontrol* 48: 307-319.

Keppanan, R., Sivaperumal, S., Aguila, L. C. R., Hussain, M., Bamisile, B. S., Dash, C. K., and Wang, L. 2018. Isolation and characterization of *Metarhizium anisopliae* TK29 and its mycoinsecticide effects against subterranean termite, *Coptotermes formosanus*. *Microbial Pathogenesis* 123: 52-59.

Kernasa, N., Uraichuen, S., and Kamata, N. 2016. Phylogenetic variation of the green muscadine fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin and its virulence to larvae of the sugarcane longhorn stem borer, *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae). *Agric. Nat. Resour*. 50: 421-431.

Kitamoto, Y., Suzuki, A., and Yamanaka, S. S. K. 2002. A new method for the preservation of fungus stock cultures by deep-freezing. *Mycoscience* 43: 143-149.

Klingen, I., Eilenberg, J., and Meadow, R. 2002. Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agric. Ecosyst. Environ*. 91: 191-198.

Kmitowa, K., Bajan, C., Wojciechowska. 1977. Differences in the pathogenicity of entomopathogenic fungi from France and Poland. *Pol. Ecol. Stud*. 3: 115-126.

Komaki, A., Kordali, S., Bozhuyuk, A. U., Altinok, H. H., Kesdek, M., and Altinok, M. A. 2017. Laboratory assessment for biological control of *Tribolium*

confusum du Val., 1863 (Coleoptera: Tenebrionidae) by entomopathogenic fungi. *Turkish J. Entomol.* 41(1): 95-103.

Koruth, A., Kumary, S. L., and Sreekumaran, V. 2014. *Three Decades of Soil Science Research in Kuttanad and Pokkali*. Kerala Agricultural University Press, Mannuthy, 72p.

Kotwal, S., Parate, R. L., Mane, S. S., and Deshmukh, V. V. 2012. Influence of nutrition and different physical parameters on growth and sporulation of *Metarhizium anisopliae*. *Int. J. Sci. Environ. Technol*. 1(5): 479-484.

Lastra, C. C. L., Hajek, A. E., and Humber, R. A. 2002. Comparing methods of preservation for cultures of entomopathogenic fungi. *Can. J. Bot*. 80: 1126-1130.

Liu, S. F., Ye, Z. H., and Jiang, S. R. 2007. Isolation and virulence test of *Metarhizium*. *J. Anhui. Agric. Sci*. 35(17): 5058-5059.

Liu, L., Zhan, R., Yang, L., Liang, C., Zeng, D., and Huang, J. 2012. Isolation and identification of *Metarhizium anisopliae* from *Chilo venosatus* (Lepidoptera: Pyralidae) cadaver. *Afr. J. Biotechnol*. 11(30): 7609-7617.

Liu, Z. Y., Milner, R. J., McRae, C. F., and Lutton, G. G. 1993. The use of dodine in selective media for the isolation of *Metarhizium* spp. from soil. *J. Invertebrate Pathol.* 62(3): 248-251.

Lomer, C. H. and Lomer, C. S. 1996. *Laboratory Techniques in Insect Pathology*. Lubilosa Tech. Bull. No.3, CABI Bioscience, UK, 38p.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem*. 193: 265-275.

Machwe, A., Senzik, A. M., and Kapoor, M. 2002. Induction profiles and properties of a novel stress induced peroxidase in *Neurospora crassa*. *Mycoscience* 43: 103-111.

Magan, N. 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. In: Butt, T. M., Jackson, C., and Magan, N. (eds), *Fungi as Biocontrol Agents*. CAB International, UK, pp. 239-257.

Mahadevan, A. and Sridhar R. 1986. *Methods in Physiological Plant Pathology*. Sivakami Publishers, Madras, pp.103-104.

Matawele, P., Trinci, A. P. J., and Gillespie, A. T. 1994. Mutants of entomopathogenic fungi that germinate and grow at reduced water activities and reduced relative humidities are more virulent to *Nephotettix virescens* (green leafhopper) than the parental strain. *Mycol. Res.* 98: 1329-1333.

Meyling, N. V. and Eilenberg, J. 2006. Isolation and characterization of *Beauvaria bassiana* isolates from phylloplanes of hedgerow vegetation. *Mycol. Res*. 110:188-195.

Mochi, D. A., Monteiro, A. C., and Barbosa, J. C. 2005. Action of pesticides to *Metarhizium anisopliae* in soil. *Neotropical Entomol*. 34: 961-971.

Mochi, D. A., Monteiro, A. C., Bortoli, S. A., Doria, H. O. S., and Barbosa, J. C. 2006. Pathogenicity of *Metarhizium anisopliae* for *Ceratitis capitata* (Wied.) (Diptera: Tephritidae) in soil with different pesticides. *Neotropical Entomol*. 35(3): 382-389.

Miller, C. D., Rangel, D., Braga, G. U., Flint, S., Kwon, S. I., Messias, C. L., Roberts, D. W., and Anderson, A. J. 2004. Enzyme activities associated with oxidative stress in *Metarhizium anisopliae* during germination, mycelial growth and conidiation and in response to near-UV irradiation. *Can. J. Microbiol.* 50(1): 41-49.

Milner, R. J., Lutton, G. G., and Zouyi, L. 1992. Methods for isolating *Metarhizium anisopliae* from soil. *Annu. Meeting Soc. Invertebrate Pathol*. 176p.

Mohan, A. and Sreelatha, A. K. 2016. Nutrient dynamics in Pokkali soils. *Int. J. Appl. Pure Sci. Agric*. 9(2): 119-123.

Namasivayam, S. K. R., Aarthi, R., and Anbazhahan, P. 2015. Studies on factors influencing the viability of entomopathogenic fungi *Metarhizium anisopliae* in soil adapting culture dependent method. *J. Biopestic.* 8(1): 23.

Neuman, G. and Shields, E. J. 2004. Survey for entomopathogenic nematodes and fungi in alfalfa snout beetle infested fields in Hungary and in New York state. *Great Lakes Entomol*. 37: 152-158.

Neves, P. M., Hirose, E., Tchujo, P. T., and Moino, J. R. A. 2001. Compatibility of entomopathogenic fungi with neonicotinoid insecticides. *Neotropical Entomol.* 30(2): 263-268.

Nussenbaum, A. L., Lewylle, M. A., and Lecuona, R. E. 2013. Germination, radial growth and virulence to boll weevil of entomopathogenic fungi at different temperatures. *World Appl. Sci. J.* 25(8): 1134-1140.

Onions, A. H. S. 1983. Preservation of fungi. In: Smith, J. E. and Jayaraj, S. (eds) *The Filamentous* Fungi. Edward Arnold London, pp. 373-390.

Ouedraogo, A., Fargues, J., Goettel, M. S., and Lomer, C. J. 1997. Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride*. *Mycopathologia* 137(1): 37-43.

Padin, S. B., Bello, G. M., and Vasicek, A. L. 1997. Pathogenicity of *Beauveria bassiana* for adults of *Tribolium castaneum* (Coleoptera: Tenebrionidae) in stored grains. *Entomophaga* 42(4): 569-574.

Padmini, P. P. C. and Padmaja, V. 2014. Defensive role of antioxidant enzymes peroxidase, esterase and catalase in *Beauveria* species under abiotic stress stimuli. *J. Chem. Biol. Phys. Sci*. 4: 1196-1208.

Parkinson, D., Gray, T. R., and Williams, S. T. 1971. *Methods for Studying the Ecology of Soil Micro-organisms*. Oxford, Blackwell Scientific Publications, New Jersey. 116p.

Patil, S. D., Kadam, J. R., Chandele, A. G., Wagh, S. S., and Jadhav, R. S. 2014. Growth, development and viability of *Metarhizium anisopliae* on media with various nutrient sources. *Int. J. Plant Prot.* 7(2): 420-423.

Paul, J. S., Tiwari, K. L., and Jadhav, S. K. 2015. Long term preservation of commercial important fungi in glycerol at 4°C. *Int. J. Biol. Chem*. 9(2): 79-85.

Posadas, J. B., Comerio, R. M., Mini, J. I., Nussenbaum, A. L., and Lecuona, R. E. 2012. A novel dodine-free selective medium based on the use of cetyl trimethyl ammonium bromide (CTAB) to isolate *Beauveria bassiana*, *Metarhizium anisopliae* sensu lato and *Paecilomyces lilacinus* from soil. *Mycologia* 104(4): 974-980.

Poosapati, S., Ravulapalli, P. D., Tippirishetty, N., Vishwanathaswamy, D. K., and Chunduri, S. 2014. Selection of high temperature and salinity tolerant *Trichoderma* isolates with antagonisitic activity against *Sclerotium rolfsii*. *SpringerPlus* 3(1): 641.

Quintela, E. D. and McCoy, C. W. 1998. Synergistic effect of imidacloprid and two entomopathogenic fungi on the behaviour and survival of larvae of *Diaprepes abbreviates* (Coleoptera: Curculionidae) in soil. *J. Econ. Entomol.* 91(1): 110-122.

Rachappa, V., Lingappa, S., and Patil, R. K. 2009. Growth characteristics and bioefficacy of different isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin against certain key insect pests. *J. Biol. Control* 23(3): 271-276.

Raid, R. N. and Cherry, R. H. 1992. Effect of soil parameters on pathogenicity of the fungus *Metarhizium anisopliae* to the sugarcane grub *Ligyrus subtropicus* (Coleoptera: Scarabaeidae). *Fla Entomologist* 75(2): 179-184.

Rangel, D. E., Braga, G. U., Anderson, A. J., and Roberts, D. W. 2005. Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *J. Invertebrate Pathol.* 88(2): 116-125.

Rangel, D. E., Alston, D. G., and Roberts, D. W. 2008. Effects of physical and nutritional stress conditions during mycelial growth on conidial germination speed, adhesion to host cuticle, and virulence of *Metarhizium anisopliae*, an entomopathogenic fungus. *Mycol. Res.* 112(11): 1355-1361.

Reddy, G. B., Sukumar, K., Kumar, K. R., Swarnabala, G., Reddy, K. V., and Muneel, V. 2017. Economical biomass production of aerial conidia of *Metarhizium anisopliae* MCC0051 using agri residues as solid substrate and

its bioefficacy in controlling *Helicoverpa armigera*. *J. Agric. Vet. Sci*. 10(10): 42-46.

Reetha, S., Bhuvaneswari, G., Selvakumar, G., Thamizhiniyan, P., and Pathmavathi, M. 2014. Effect of temperature and pH on growth of fungi *Trichoderma harzianum*. *J. Chem. Biol. Phys. Sci*. 41: 283-292.

Reynolds, S. G. 1970. The gravimetric method of soil moisture determination part 1 and a study of equipment and methodological problems. *J. Hydrology* 11(3): 258-273.

Rishi, R. R., Borah, R. K., Rajesh, K., and Shailesh, P. 2013. Isolation, identification and mass production of soil microbes and their utility for biocontrol. *Int. J. Adv. Life. Sci*. 6: 168-173.

Rombach, M. C., Aguda, R. M., Shepard, B. M., and Roberts, D. W. 1986. Entomopathogenic fungi (Deuteromycotina) in the control of the black bug of rice, *Scotinophara coarctata* (Hemiptera; Pentatomidae). *J. Invertebrate Pathol.* 48(2): 174-179.

Roberts, D. W. and Campbell, A. S. 1977. Stability of entomopathogenic fungi. *Ann. Entomol. Soc. Am.* 10: 19-76.

Sadasivam, S. and Manickam, A. 2008. *Biochemical Methods* (3rd Ed.). New Age International Publishers, New Delhi, India, 218p.

Samuels, K. D. Z., Pinnock, D. E., and Allsopp, P. G. 1989. The potential of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) as a biological control agent of *Inopus rubriceps* (Macquart) (Diptera: Stratiomyidae). *Aust. J. Entomol*. 28(1): 69-74.

Samson, R. A., Evans, H. C., and Latge, J-P. 1988. *Atlas of Entomopathogenic Fungi*. Springer, Verlag, Berlin, 187p.

Sasan, R. K. and Bidochka, M. J. 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. *Am. J. Bot*. 99(1): 101-107.

Sepulveda, M., Vargas, M., Gerding, M., Ceballos, R., and Oyarzua, P. 2016. Molecular, morphological and pathogenic characterization of six strains of *Metarhizium* spp. (Deuteromycotina: Hyphomycetes) for the control of *Aegorhinus superciliosus* (Coleoptera: Curculionidae). *Chilean J. Agric. Res*. 76(1): 77-83.

Silva, A. M. M. D., Borba, C. M., and Oliveira, P. C. D. 1994. Viability and morphological alterations of *Paracoccidioides brasiliensis* strains preserved under mineral oil for long periods of time. *Mycoses* 37: 165-169.

Singer, M. A. and Lindquist, S. 1998. Multiple effects of trehalose on protein folding *in vitro* and *in vivo*. *Mol. Cell* 1: 639–648.

Smith, D. 1993. Long-term preservation of test strains (fungus). *Int. Biodeterioration Biodegradation* 31(3): 227-230.

Sowmya, G. 2017. Collection, isolation and screening of entomopathogenic fungi, *Metarhizium anisopliae* (Metchnikoff) Sorokin. MSc(Ag) thesis, University of Horticultural Sciences, Bagalkot, 72p.

St Leger, R. J., Joshi, L., and Roberts, D. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. *Appl. Environ. Microbiol*. 64(2): 709-713.

St Leger, R. J., Nelson, J. O., and Screen, S. E. 1999. The entomopathogenic fungus *Metarhizium anisopliae* alters ambient pH, allowing extracellular protease production and activity. *Microbiology* 145(10): 2691-2699.

Sujatha, K. and Padmaja, V. 2014. Alterations in electrophoresis patterns of proteins, isozymes and *in vitro* protease activity under thermal stress in two isolates of *Metarhizium anisopliae*. *Ann. Plant Sci*. 3(10): 845-853.

Sun, B. D., Han, Y. Y., Ananda, J. C., and Xing- Zhang, L. 2008. Insect associated fungi in soils of field crops and orchards. *Crop Prot*. 27: 1421-1426.

Sun, X., Yan, W., Oin, W., Zhang, J., Niu, X., Ma, G., and Li, F. 2016. Screening of tropical isolates of *Metarhizium anisopliae* for virulence to the red palm

weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae). *Springerplus* 5(1): 1100.

Tangthirasunun, N., Poeaim, S., Soytong, K., Sommartya, P., and Popoonsak, S. 2010. Variation in morphology and ribosomal DNA among isolates of *Metarhizium anisopliae* from Thailand. *J. Agric. Technol*. 6(2): 317-329.

Tanzini, M. R., Alves, S. B., Setten, A. 2002. Toxicity of phytosanitary products used for the control of *Leptopharsa heveae* to entomopathogenic fungi, *Arquivos do Instituto biologica* 69: 65-69.

Teja, K. N. P. C and Rahman, S. J. 2016. Characterisation and evaluation of *Metarhizium anisopliae* (Metsch.) Sorokin strains for their temperature tolerance. *Mycology* 7(4): 171-179.

Teja, K. N. P. C and Rahman, S. J. 2017. Effect of media pH on the growth of entomopathogenic fungi isolated from different rhizosphere soils. *Int. J. Bioassays* 6(3): 5325-5327.

Thevelein, J. M. 1984. Regulation of trehalose mobilization in fungi. *Microbiol. Rev*. 48(1): 42–59.

Toegel, S., Salar-Behzadi, S., Horaczek-Clausen, A., and Helmut, V. 2010. Preservation of aerial conidia and biomasses from entomopathogenic fungi *Beauveria brongniartii* and *Metarhizium anisopliae* during lyophilisation. *J. Invertebrate Pathol*. 105: 16-23.

Tulloch, M. 1976. The genus *Metarhizium*. *Trans. Br. Mycol. Soc*. 66(3): 407-411.

Ummidi, V. R. S., Josyula, U., and Vadlamani, P. 2013. Germination rates of *Beauveria bassiana* and *Metarhizium anisopliae* its possible correlation with virulence against *Spodoptera litura* larvae. *Int. J. Adv. Res*. 2: 625-630.

van Asperen, K. 1962. A study of housefly esterases by means of a sensitive colorimetric method. *J. Insect Physiol*. 8(4): 401-416.

Vanninen, I. 1995. Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. *Mycol. Res.* 100: 93-101.

Vanninen, I. and Hokkanen, H. 1988. Effects of pesticides on four species of entomopathogenic fungi. *Ann. Agriculturae Fenniae* 27: 345-353.

Veen, K. H. and Ferron, P. 1966. A selective medium for isolation of *Beauveria tenella and Metarhizium anisopliae*. *J. Invertebr. Pathol*. 8: 268-269.

Vega, F. E., Goettel, M. S., Blackwell, M., Chandler, D., Jackson, M. A., Keller, S., Koike, M., Maniania, N. K., Monzon, A., Ownley, B. H., Pell, J. K., Rangel, D. E. N., and Roy, H. E. 2009. Fungal entomopathogens: new insights on their ecology. *Fungal Ecol*. 2(4): 149-159.

Vincent, J. M. 1927. Distortion of fungal hyphae in the presence of certain inhibitors. *Nature* 59: 850.

Wakil, W., Ghazanfar, M. U., and Yasin, M. 2014. Naturally occurring entomopathogenic fungi infecting stored grain insect species in Punjab, Pakistan. *J. Insect Sci.* 14(182): 1-7.

Wang, X., Zhang, Y., Wang, C., Wang, C., and Hou, L. 2013. Intracellular level of trehalose in soy sauce yeasts under different stresses. *Int. Conf. Agric. Nat. Resour. Eng*. 5: 321-326.

Windels, C. E., Burnes, P. M., and Kommedahl, T. 1993. *Fusarium* species stored on silica gel and soil for ten years. *Mycologia* 85(1): 21-23.

Wyrebek, M., Huber, C., Sasan, R. K., and Bidochka, M. J. 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiol*ogy 157(10): 2904-2911.

Yewale, A. C. 2001. Multiplication and use of *Metarhizium anisopliae* as biocontrol agent against *Spodoptera litura*. MSc(Ag) thesis, PGI, DR.PDKV, Akola, (UnPub).

Zimmermann, G. 1986. The ‘Galleria bait method’ for detection of entomopathogenic fungi in soil. *J. Appl. Entomol*. 102(1-5): 213-215.

Abstract

SELECTION FOR ABIOTIC STRESS TOLERANT ISOLATES OF *Metarhizium anisopliae* SOROKIN

by

JANCY MERLIN JOHNSON

(2017-11-008)

ABSTRACT OF THE THESIS

Submitted in partial fulfillment of the requirement for the degree of

Master of Science in Agriculture

(AGRICULTURAL ENTOMOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL ENTOMOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2020

Selection for abiotic stress tolerant isolates of *Metarhizium anisopliae* Sorokin

Abstract

Metarhizium anisopliae Sorokin, is a widely used entomopathogenic fungus for the biocontrol of insect pests. However, vulnerability to various abiotic stresses limits its performance as a biological control agent. Identification and evaluation of isolates tolerant to abiotic stresses can help overcome this limitation. To this end, soil samples were collected through purposive sampling from areas in central Kerala with a history of abiotic stresses. *Metarhizium anisopliae* isolates were obtained from soil samples collected from Moncompu, Alappuzha among the areas surveyed. Three isolates *viz*., MC 2, MC 4 and MC 7 were evaluated for tolerance to acidity, salinity, high temperature, drought, insecticides and fungicides.

Highest acidity tolerance was recorded by the isolate MC 7 with the highest mycelial biomass of 1.15 g at the lowest pH of 2.5. In the salinity tolerance trials, isolate MC 2 exhibited highest growth and sporulation at 0.5 M NaCl with only 6.33 per cent growth inhibition while the other two isolates recorded more than 50 per cent growth inhibition. At the higher salt concentration of 1.5 M, more than 89 per cent growth inhibition was recorded in case of isolates MC 4 and MC 7, while MC 2 recorded the least inhibition of 58.84 per cent indicating high salinity tolerance.

Screening for temperature tolerance at temperatures ranging from 25 to 40°C revealed that the isolate MC 7 recorded highest mycelial biomass (1.28 g) at 37°C. Similarly, studies on drought tolerance showed that the isolate MC 2 survived at highest concentration of 35 per cent Polyethylene glycol (PEG) with mycelial weight of 0.93 g suggesting drought tolerance as well. The isolate MC 2 recorded multiple stress tolerance and was found to be suited to both saline and drought prone areas. Similarly, the isolate MC 7 was found to be highly suitable to acidic soils as well as high temperature areas.

Compatibility of *M. anisopliae* isolates with popular insecticides like spinosad, cypermethrin, imidacloprid and chlorantraniliprole as well as fungicides like copper oxychloride, carbendazim and hexaconazole was also assessed. All the isolates showed less than 22 per cent growth inhibition in PDA medium amended with the highest doses of pesticides except

cypermethrin, carbendazim and hexaconazole. Based on the radial growth and sporulation, isolates MC 7 and MC 2 were found to be relatively more compatible to both insecticides and fungicides.

Biochemical analysis of the stress tolerant isolates, MC 7 and MC 2 revealed that the defensive enzymes like peroxidase and catalase were elevated both at highest temperature (37°C) and highest concentration of PEG (35 %). Both the isolates exhibited peroxidase activity of 0.058 and 0.030 EU/min/g tissue and catalase activity of 0.140 and 0.294 EU/min/mg protein respectively when compared to control. Quantity of trehalose was also maximum at highest temperature and at highest concentration of PEG with a value of 1.380 (MC 7) and 1.580 mg/ml/g mycelia (MC 2) respectively. Esterase, an enzyme essential for fungal infection process was also estimated and its activity was low in the isolate MC 7 at 37°C as well as in the isolate MC 2 at 35 per cent PEG when compared to respective control treatments.

Biocontrol efficacy of *M. anisopliae* isolates was evaluated against both adults and grubs of the storage pest, *Tribolium castaneum* at different doses of 1×10^5 to 1×10^9 spores/ml under laboratory conditions. The lowest LT_{50} values of 9.43 days and 4.14 days were recorded by the isolate MC 2 at a concentration of 1×10^9 spores/ml on adult and grub respectively. However, LT_{50} values were on par with that of positive check, NBAIR strain as well as MC 7.

The study was successful in identifying isolates of *M. anisopliae* with potential for biocontrol in agroecosystems vulnerable to abiotic stresses. It also helped to highlight the importance of screening potential stress tolerant isolates of microbial bioagents.