NOVEL STRAINS OF *Metarhizium anisopliae* SOROKIN. (ASCOMYCOTA: SORDARIOMYCETES) WITH ENHANCED ABIOTIC STRESS TOLERANCE

by

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(2020-11-041)

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KERALA, INDIA

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THESIS

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2023

DECLARATION

I hereby declare that this thesis entitled "**Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance**" 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.

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CERTIFICATE

Certified that this thesis entitled "**Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance**" is a bonafide record of research work done independently by Ms. Sreelakshmi U. K. (2020-11-041) 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.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

1. INTRODUCTION

Biological insecticides offer a range of environmentally acceptable choices for the cost-effective management of insect pests in response to growing public concern over the continuous use of synthetic chemical insecticides. The entomopathogenic green muscardine fungi, *Metarhizium* spp., is employed as an environmentally beneficial substitute for chemical insecticides for managing a number of pests. More than 100 insects, including several soil-dwelling insects, have been found to be infected by the entomopathogenic fungus *M. anisopliae*. Many biopesticides that are currently in the market exhibit poor performance in the field. The entomopathogenic fungi (EPF) are negatively impacted by changing abiotic variables, including changing temperatures, water availability, UV radiation, and rainfall events. The performance and longevity of a fungal formulation in the field mostly depend on conidial adaptability to adversities (Li and Feng, 2009). The conidial germination and sporulation of EPF can be delayed and become less effective as a result of thermal stress and protracted drought. Over the last century, the average global temperature had increased by 10^{o}C and by 2100, the temperature is expected to reach 40^{o}C. Majority of the mycopesticide research has focused on increasing propagule yield, with little to no attention paid to the quality of inoculum. This is a major impediment to the effective commercialization of mycopesticides in actual field settings.

During summer, temperature of the storage houses of fungal products reach up to 40 ^{o}C or more and packing or stacking of these products also worsen the situation. Transportation of mycopesticides under high temperature conditions also results in loss of spore viability. Newer strains of EPF having tolerance to such abiotic fluctuations are a prerequisite for the successful development of competent biocontrol agents for use in the field. Unravelling the biochemical mechanisms of stress tolerance in EPF will help to develop newer potential biocontrol agents for field use.

Potential strains of *M. anisopliae* that are tolerant to abiotic stresses and those thrive even in the raised temperature conditions are to be developed for use in pest management. Hence, it is suggested to develop *M. anisopliae* strains for use in pest control that can withstand abiotic challenges and flourish at elevated temperatures. Therefore, the current study aims to generate strains of the EPF, *M. anisopliae*, that

are resistant to heat and drought for the successful control of damaging pests in the field. The study includes the following objectives:

- ✓ Inducing temperature tolerance in *Metarhizium anisopliae* Sorokin strains
- ✓ Inducing drought tolerance in *Metarhizium anisopliae* Sorokin strains
- ✓ *In vitro* screening of *Metarhizium anisopliae* Sorokin strains for biocontrol efficacy
- ✓ Biochemical analysis of stress tolerance and virulence

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Due to the growing need for transition to sustainable agricultural practices, the use of biocontrol agents in pest and disease management has been tremendously increased during the past few years. Entomopathogenic fungi provide a better and safer alternative to chemical insecticides and pesticides, which are associated with environmental and human health risks. The anamorphic species, *Metarhizium anisopliae* is a potential entomopathogenic fungus for future biological control. This study entitled "Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance" aims to develop improved strains of *M. anisopliae* with stress tolerance. A scrutiny of the literature in connection with the study is presented in this chapter.

2.1. Characterization of native isolates of *Metarhizium anisopliae*

According to Gams and Rozsypal (1973), *M. anisopliae*, the fungus that causes green muscardine is a polyphagous, global species that is dark green with widey varying conidial sizes. Colonies of *M. anisopliae* var. *anisopliae* can be of any shade of green and contain cylindrical to oval conidia that are often truncate at both ends and measure 3.5-9.0 μm (typically 5.0-8.0 μm) in length (Tulloch, 1976). Zimmerman (1986) mentioned that the green, cylindrical conidia that are generated in chains and form a dense, compact covering of spores.

The conidia are cylindrical with round ends that vary in colour from pale green to dark green and have clavate phialides containing dull brown conidia (Latch *et al.,* 1964). Colonies grow on oatmeal agar and malt extract, initially white, then greyish yellow-green, and finally pale olivaceous. Conidial columns have caused a mealy, smooth, or granular buff surface (Rayner, 1970). As per the descriptions made by Bridge (2004), *M. anisopliae* has a buff that is yellowish green, olivaceous, dark herbage green, pink, or vinaceous. Conidia are formed in chains and the ends of the phialides may be slightly swollen (Milner *et al.*, 2002).

Fernandes *et al.,* (2010) identified that the conidia of *M. anisopliae* are cylindrical to oval, somewhat constricted in the middle, typically truncate at both ends, and measure between 3.5 and 9 μm in length and produce colonies in a variety of hues of green, sepia, or isabelline. Tangthirasunun (2010) mentioned that the

conidiophores are straight forward and branched apical phialides to create conidia and grow on phialides that extend from the septate mycelium.

2.2. Sub culturing of *Metarhizium anisolpiae*

Hall (1980) in his study pointed out that, despite modifications to the shape and rates of growth, single and multispore isolates showed stability in virulence following all subcultures. According to Ignoffo *et al.,* (1982), if the pathotype is stable and the media and growing conditions used are optimum then a decrease in virulence should not be there after repeated sub culturing the entomopathogenic fungus. Reviving the EPF through a live insect host is one of the most popular ways to boost or restore the lost virulence (Hayden, 1992). The development morphology and conidiation traits of the several generations did not appear to differ qualitatively. At the time of harvest, the spore production following each sub-culturing was roughly the same (Vidal *et al.,* 1997a; Fargues *et al.,* 1997).

Thomas and Jenkins (1997) recently noted that in *M. flavoviride*, producing submerged conidia, the number, and quality of blastospores can be influenced by the culture conditions and vary between strains. The development morphology and conidiation traits of the several passages did not appear to differ qualitatively and also the spore production following each passage was roughly the same (Brownbridge *et al.,* 2001). In a research conducted by Brownbridge *et al*., (2001), they claimed that the pathogenicity of fungus subcultured in artificial media did not decrease. Higher virulence has been linked to faster germination in *Paecilomyces fumosoroseus*.

Hutwimmer *et al.,* (2008) found that there is only a little information that fungus attenuates, but it is clear that different strains have varying levels of stability when cultivated on the artificial substrate. Ansari and Butt (2010) cited that unstable strains often show a decrease in virulence after a few subcultures but stable strains maintain their virulence for numerous generations.

2.3. *Invitro* screening of *Metarhizium anisopliae* isolates for temperature tolerance

Different ways that high temperatures might affect cells include protein denaturation and disorganization of the membrane (Setlow and Setlow, 1995). The most significant abiotic parameters impacting the survival and infectiousness of EPF are solar radiation, humidity, and temperature (Bugeme *et al.,* 2008; Inyang *et al.,*

2000). The screening experiments conducted by Borisade and Magan, (2015) proved that *M. anisoplaie* strains showed tolerance to higher temperature. Oliveira *et al.,* (2018) mentioned that temperatures exceeding 30 °C are frequently experienced during the spring and summer in tropical regions, which may affect the vigor and survival of conidia.

The rate of *in vitro* conidial germination of some *M. anisopliae* isolates was slower at 10 and 15^{o}C than at 20 and 25^{o}C and the greatest reduction in rates of conidial germination and colony growth was observed at 10^{o}C, compared with other temperatures (Yeo *et al.,* 2003). As per Nussenbaum *et al.,* (2013) and Vidal *et al*., (1997b), the rate of conidial germination and mycelial development, which is directly related to the rate of infection and virulence against the target pests, is primarily influenced by temperature. Fungal entomopathogens often thrive between the temperatures of 23^{o}C and 28 ^{o}C but fail at 34^{o}C to 37^{o}C (Jaronski, 2009).

Walstad *et al.,* (1970) reported that *Beauveria bassiana* and *M. anisopliae* initiate germination even at a higher temperature range of 35^{o}C. Studies on the effects of temperature on *Metarhizium* species revealed that the majority of them could grow at temperatures between 11 and 32 °C (Ouedraogo *et al.,* 1997). In lab tests on an *M. anisopliae* strain conducted by Ekesi *et al*., (1999), the temperature range between 25 and 30 ^{o}C yielded the fastest growth rates. Mouchacca (2000) studied that *in vitro* mycelial growth of *M. anisopliae* is influenced by temperature.

2.3.1. Thermotolerance in *Metarhizium anisopliae* isolates

The maximum temperature for mycelial growth for the insect-pathogenic fungus *Metarhizium anisopliae* is 37- 40°C (Hallsworth and Magan, 1999; Thomas and Jenkins, 1997). The green mauscardine fungus, *M. anisopliae* has a stronger thermotolerance than other species (Horaczek and Viernstein, 2004; Rangel *et al.,* 2005). According to St. Leger *et al*., (2001), the maximum temperature for conidial germination and mycelial growth of *M. anisopliae* is approximately 35–37°C. The thermal death point for *M. anisopliae* conidia has been determined to be between 49 and 60°C.

Vidal, (1997a) found that, compared to populations of the same species living at different latitudes, fungal populations from the equator can withstand higher temperatures. The heat tolerance of fungi and other eukaryotic organisms is

significantly different from that of prokaryotes and archaea, and most fungi have limited growth at temperatures above 55°C (Rangel *et al*., 2005).

The majority of insect-damaging fungi are mesophilic, with growth optimum temperatures between 25 and 35°C and a growth range between 10 and 40°C (Cooney and Emerson, 1964). The thermal death point of *M. anisopliae* spores was estimated by Vouk and Klas (1932) and found out to be between 55°C and 60°C. Only a few species of fungi, including both thermophilic and thermotolerant varieties, are eukaryotic creatures that can survive in the range of 45 to 55°C (Maheswari *et al*., 2000). Rangel *et al*. (2005) demonstrated that *M. anisopliae* conidial thermotolerance was observed at temperatures of 40 or 45^{o}C.

Ouedraogo *et al*., 1997 mentioned that, most of the isolates of *Metarhizium* spp. preferred a temperature of 25^{o}C for growth. Hedgecock *et al.,* (1995) and Rangel *et al.,* (2005) found that the conidial viability may be affected by temperature in a variety of ways, including incubation period, conidial moisture content, and volume of the heated suspension. When conidial suspensions were exposed to higher and lower temperatures, *M. anisopliae* isolates showed a significant variation in their thermotolerance (Fernandes *et al.,* 2015). Strains that are more thermotolerant have a better chance of adjusting to the summer when agricultural infestations by insect pests are typically at their peak (Li and Feng, 2009).

2.4. *Invitro* screening of *Metarhizium anisopliae* isolates for drought tolerance

Schaerffenberg (1963), malt peptone yeast (MPY) agar was used to study how moisture affects spore germination. Hussein *et al.,* (2010) found that the amount of moisture present in soil has a significant impact on survival, reproduction and infection of the host. Several environmental parameters, including soil moisture, air and soil temperatures, air relative humidity, and solar UV radiation, have an impact on growth and establishment of *M. anisopliae* (Chen *et al*., 2014).

According to the study of Hallsworth and Magan (1994), conidia of entomopathogenic fungi are not able to germinate under low level of moisture content. According to Carpenter and Crowe (1988) and Brown (1978) Polyols are known to accumulate to high concentrations in fungus at low water availability, and also prevent enzyme inhibition from dehydration. As per Livingston (1993), the osmotic potential of soil is produced by dissolved solutes and decreased water activity as a result of

contact with charged surfaces. There is a significant impact on the fungi when the water stress is manipulated by changing the carbon concentration in the media (Ramos, 1999).

Zidan and Abdel-Mallek (1987) discovered that intracellular glycerol accumulation by *Aspergillus ochraceous A. niger and A. tamari* greatly increased when the osmotic potential was altered to around -70 MPa, but decreased at an osmotic potential of -7 to -10 MPa. In general, it has been shown that fungal germination and growth are more responsive to osmotic potential stress (Brownwell and Schneider, 1983; Magan, 1988; Challen and Elliot, 1986). Typically, medium adapted to varied water activities are used to study the effects of moisture stress on germination, development, and sporulation of entomopathogenic Hypocreales (Inglis *et al*., 2012).

When water from a culture evaporates, the water activity of the medium gradually declines and may finally reach a level that inhibits the growth of fungi (Inch and Trinci, 1987). In a study conducted by Ramos *et al.,* (1999), they observed a wide range of solute potential (-0.75 to -28 MPa) for the generation of cleistothecia.

2.4.1. Drought tolerance

The patterns of accumulation of polyols and trehalose in both nonxerophilic and xerophilic fungi are significantly influenced by osmotic stress alone, as well as interactions with temperature and time (Luard, 1982; Hocking and Norton, 1983; Hocking 1993 and Hallsworth and Magan, 1995). Trehalose and polyhydroxy alcohols (polyols) may be crucial for propagule survival during desiccation and successful germination (Al-Hamdani and Cooke, 1987; Harman *et al.,* 1991; Gornova *et al.,* 1992). Magan (2001) identified that the xaerotolerant fungi will produce low molecular weight substances like polyols, sugars, alcohols, and glycerol under moisture stress to overcome the stress.

If osmotic mechanisms govern water potential, a decline in water potential is linked to an increase in solute concentration, which raises the possibility of nutritional imbalances, particular ion effects and growth-inhibiting effects (Adebayo and Harris, 1971). Matewele (1994) suggested that increased endogenous concentrations of polyols may be connected to the ability of mutants of *Paecilomyces farinosa and M. anisopliae* to grow at water activities that impede the development of the parental

strains. As per Ramos *et al.,* (1999), the main polyol accumulated in mycelium was typically glycerol, with lesser amounts of arabitol and erythritol, and at lowered solute potential glycerol levels rose whereas mannitol levels were frequently decreased.

The intracellular build up of these polyols lowers cytoplasmic water activity without impairing the structure and operation of the enzymes, allowing metabolic activity to continue even when there is a lack of water (Brown, 1978). Hallsworth and Magan (1994) in their study mentioned that conidia of *B. bassiana, M. anisopliae,* and *P. farinosa* accumulated glycerol and erythritol when cultures were grown at a water activity of less than 0.97 Mpa or supplied with a lot of glycerol. Laboratory research suggests that conidia with high glycerol and erythritol concentrations kill insects more quickly and at lower relative humidity (RH) than normal propagules (Hallsworth and Magan, 1994). According to the *in vitro* research conducted by Magan (1988), fungi that were given a medium with altered osmotic and matric potentials had significantly different propensities for germination and growth.

2.5. Preserving *Metarhizium anisolpiae* isolates

Cavalcanti (1991) and Silva *et al.,* (1994) noted that cultures submerged in mineral oil may endure for decades. Pathogenicity of entomopathogens may persist even months after storage (Balardin and Loch, 1988). The simplest method of preservation may be storing metabolically inert fungi in sterile distilled water, which is effective for a wide variety of fungi including human or plant pathogens (Castellani, 1967; Figueiredo and Pimentel, 1975).

Standard home freezers might seem to be a perfect and affordable method to maintain microbial cultures and various fungal cultures were successfully preserved at 20^{o}C by Carmichael (1962). Smith (1993) found that aerobic microorganisms that frequently use 20^{o}C freezers can only be stored using sterile, anhydrous silica gel crystals. Bell and Hamalle (1974) also supported this by citing that it is one of the most inexpensive ways of fungal preservation. Fungi stored in silica gel will stay viable for up to 25 years (Sharma and Smith, 1999).

Because of its ease of use, dependability, and simplicity, the majority of mycological laboratories that freeze fungi employ only 10 percent glycerol (Sanskar and Magalhaes, 1994). Screwcap cryovials are very expensive for cryopreservation and an alternative to this is the sections of polypropylene drinking straws with heat-

sealed ends, which can be securely submerged in liquid nitrogen. (Stalpers *et al.*, 1987; Challen and Elliott, 1986).

According to a method created by Homolka *et al.,* (2010) for freezing basidiomycete fungus, cultures can be grown on sterile perlite which is moistened with wort broth and given 5 per cent glycerol as a cryoprotectant. Another quick and low-cost technique that works with conidial entomopathogens is growing fungal cultures on pieces of sterile filter paper on an agar plate (Fong *et al.,* 2000).

2.4. Induction of temperature tolerance to *Metarhizium anisopliae* isolates

According to Herbert and Bhakoo (1979), one of the main variables restricting the growth of all microorganisms is temperature, hence it is important to understand how well these organisms can survive in various temperature ranges in order to maximize their economic utility. According to Liu *et al.,* (2003), when mycelia grow under thermal stress conditions the EPF tolerates heat by stimulating high-protein metabolism. Temperature is a critical factor that affects EPF pathogenicity, growth, and survival (Lekime *et al*, 2008). It is shown that behavioral alterations are sufficient to increase stress in pathogens to attenuate the infection process when insects infected with fungi expose themselves to a behavioral fever by choosing hotter surroundings than their normal choice (Hunt and Charnley 2011; Anderson *et al.,* 2013). As per Velavan *et al.,* (2022), there was considerable variation in conidia thermotolerance across the *Metarhizium* isolates.

Fungi have been found to have a variety of strategies for dealing with heat stress (Butler and Day, 1998).The majority of studies on temperature tolerance concentrate on conidial survival, such as thermal death thresholds or relative germination rates following heat exposure (Arthurs and Thomas, 2001). Foster *et al.,* (2011) noted that variable temperatures beyond the ideal growing range of the isolate were the main cause of the subpar performance in the field.

Ruel and Ayers (1999), found that exposing to an ideal temperature to a short time, followed by a brief period of exposure to high temperature has shown less inhibition on their growth. When it comes to infection as well as the creation and storage of mycopesticides, genetic resistance to desiccation and temperature extremes would be a clear advantage (St. Leger and Screen, 2001). As per Keyser *et al.,* (2014), the ability of entomopathogens to tolerate higher thermal stress can determine their

virulence and pathogenicity. Particularly in semi-arid areas, the capacity of some strains of EPF to grow and sporulate under a wide temperature range is especially helpful in their application as biological control agents (Teja and Rahman, 2016).

2.5. Induction of drought tolerance to *Metarhizium anisopliae* isolates

According to Ypsilos and Magan (2005), a screening of several nitrogen sources was done at a set water activity (aw) level to maximize blastospore output in a brief fermentation time. Drought and high ambient temperatures may slow down the entomopathogen proliferation and virulence (Borisade and Magan, 2014). The work done by Borisade and Magan (2015) tested the environmental toleration of five or more *B. bassiana, M. anisopliae,* and *Isaria farinosa* strains, revealed that very few strains could withstand high temperatures (35°C) and water stress (0.96 to 0.94 aw).

It has been claimed that spore germination can be improved in environments with water stress by accumulating more endogenous lower molecular weight polyols like glycerol and erythritol. This is done by reducing the intracellular water potential and facilitating faster water transport into the spore (Hallsworth and Magan, 1994a). To more accurately estimate effectiveness under drought conditions, Goettel *et al.*, (2005) advise assessing intra-specific differences in response to abiotic stress in laboratory assays before beginning field tests.

Luard and griffin (1981) found that controlled turgor may cause fungal growth, which is often reduced by lower external water potential. To maintain metabolic activity, fungi that grow in conditions with lower osmotic potential acquire low molecular weight solutes such as poly glycerol and the amino acid proline (Luard, 1982a). Hallsworth and Magan (1994b) made the argument that there is potential to manage the endogenous reserves of fungal propagates through manipulation of the growing medium, which can result in the creation of fungal propagates with higher rates of germination.

Gervais *et al.,* (1988) examined how *B. bassiana* and *M. anisopliae* grew and produced spores on a variety of peptone sources and discovered many that promoted healthy mycelial development and spore generation. Since water loss alone is unlikely to be adequate to produce such low osmotic potentials, the accumulation of one or more low molecular weight solutes through absorption or synthesis is likely necessary for the production of turgor (Luard and Griffin, 1981). Ovett (2015) stated that the

fungi which grow under low water potential conditions appear to retain a constant and high internal turgor potential.

Only a few isolates of *B. bassiana, M. anisopliae,* and *I. farinosa* were found to be able to resist elevated temperature (35^{o}C) and water stress according to Borisade and Magans (2014) environmental tolerance screening (0.94 to 0.96 water activity).

2.6. *In vitro* screening of *Metarhizium anisopliae* isolates for their biocontrol efficacy

The green muscardine fungus is a well-known entomopathogenic fungus causing diseases in a variety of insects. The virulence and pathogenicity of *M. anisopliae* can be studied using *Galleria mellonella*. It is important to prove the occurrence of an infection and the host should exhibit signs of virulence. By looking for mutants with attenuated virulence, *G. mellonella* has also proved helpful in identifying virulence determinants.

2.6.1. Suitability of *Galleria mellonella* as host

The greater wax moth, *G. mellonella* larvae are less expensive, simpler to keep, they don't need specialist labs or equipment and this is the advantage of using *Galleria* larvae as test insect for bioassay (Oliveria *et al.,* 2018). According to Lange (2018), the genome of *G. mellonella* has been sequenced, allowing for even better molecular knowledge of host-pathogen interactions and advancing the biological relevance of discoveries.

Mylonakis *et al.,* (2005) found that *G. mellonella* has been utilized as a model to discern between the relative virulence of several fungal strains at 30^{o}C and 37^{o}C. By looking for mutants with attenuated virulence, *G. mellonella* has also proved helpful in identifying virulence determinants (Slater *et al.*, 2011). As per Firacative *et al.* (2014), other fungal infections, such as *Aspergillus fumigatus, Histoplasma capsulatum, Paracoccidioides lutzii*, *Fusarium* sp., and *Cryptococcus* sp., have now been studied using *G. mellonella*.

The use of *G. mellonella* has expanded beyond the use of an entire animal system to include the preparation of cell lines to study the impact of fungal toxins on the function of immune-competent hemocytes *in vitro* in order to comprehend the mechanisms of action of toxins produced by pathogenic fungi of insects such as

cyclosporins, beauverolides, and destruxins (Vilcinskas *et al.,* 1999). Kelly and Kavanagh (2011) in their study indicated that *Galleria* larvae have been utilized as a model to investigate the differences in capacity of the biofilm-producing and non-producing isolates for their tissue invasion, pathogenicity, and the effectiveness of their antifungal agents.

The *Galleria* larvae have been used to speed the *in vivo* evaluation of possibly innovative antimicrobial treatments (Hargreaves and Clokie, 2014). Champion *et al.* (2016) mentioned that *G. mellonella* is now well-established as a useful model host for bacterial infections and for screening antimicrobial agents.

2.6.2. Efficiency of *Metarhizium anisopliae* as a biocontrol agent

As per Inglis *et al.*, (2002), biocontrol agents and entomopathogenic fungi are used in many agroecosystems to reduce pest populations and associated damages. The green muscardine fungus, *M. anisopliae*, has been linked to the development of disease in more than 200 insect pest species (Portilla and Torres, 2010). According to Anitha *et al.*, (2019), termites, thrips, and other pests are controlled by using *M. anisopliae* and related species as biological pesticides.

The ability to produce high concentrations of stable propagules at an affordable price is one of the important criteria influencing the employment of fungus in the control of agriculturally undesirable pests (Joronski, 1986). Wayal *et al,* (2018) in their study mentioned that the two most significant fungal species, *M. anisopliae,* and *B.bassiana* are the insect pathogenic fungi that are able to overcome the host resistance strateges and produce infection.

Numerous fungi that are entomopathogenic produce metabolic substances that may be poisonous to insects (Vey *et al.*, 2001). The pest management trials conducted by Sajap *et al.* (2012) proved the efficacy of *M. anisopliae* against third instar larvae of Tiger moth, (*Atteva sciodoxa*). According to Panday (2013), when compared to chlorpyriphos, *M. anisopliae* and *B. basiana* were the most effective against cutworms. Production of adhesion factors, cuticle-degrading enzymes, infection structures, and harmful secondary metabolites by the EPF will result in successful infection and spread of disease among insect pest population (Butt *et al,* 2016; Pedrini, 2018).

Samules (1989) classified 58 *M. anisopliae* wild-type isolates as extremely, moderately, and poorly pathogenic after screening them against *Nilaparvatha lugens* (Stal). The choice of fungal isolates with high virulence that exhibit considerable enzyme activities on target insects is required due to the increased interest in using EPF as pest management solutions (Gebremariam *et al.,* 2022).

The EPF, *M. anisopliae* decreased fertility, occlusion rate, preoviposition and oviposition times, egg incubation period, percentage of females ovipositing, and feeding period in *Boophilus* when applied to engorged or engorging adult female ticks (Correia *et al.,* 1998). According to Samish *et al.* (2001), *M. anisopliae* decreased the molting success of engorged *Rhipicephalus* larvae and nymphs. Singha *et al.,* (2011) discovered that *B. bassiana* and *M. anisopliae* were effective agents against tea termites (*Microtermes obesi*).

2.7. Biochemical analysis for stress tolerance and virulence

Numerous studies have been done on different defense strategies used by microbes to deal with harsh environmental circumstances. Enzymatic processes and the creation of biochemicals may be also studied.

2.7.1. Catalase activity

According to Pedrini *et al*. (2006), catalases have been related to the capacity to break down hydrocarbons present on host (insect) cuticles and these enzymes also have contributions to virulence in entomopathogenic fungi to go beyond stress response. The EPF have been shown to contain a variety of oxidative stress proteins, such as catalases, peroxidases, superoxide dismutases (SOD), and thioredoxins, as well as components of the signaling network that regulate these processes (Wang *et al*., 2013; Li *et al.,* 2015; Ortiz-Urquiza and Keyhani, 2015; Zhang *et al.*, 2016).

Jia *et al.*, (2016) mentioned that other insect enzymes that provide defense against diseases and insecticides include antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). Insect resistance to insecticides and the breakdown of toxic compounds produced during *M. anisopliae* infection are both reflected in changes in this enzyme activity, and as a result, these enzymes play a critical role in the defense of insects against infections.

According to Muller *et al.,* (2007) and Gopalakrishnan *et al.,* (2011) these enzymes can be promptly up-regulated in response to xenobiotic threats, and increases in their activity are connected to insect melanisation and pesticide resistance. As per Wang *et al.,* (2005), the most crucial enzymes for converting H_2O_2 into water and oxygen and reducing intracellular hydrogen peroxide levels are catalases and peroxidases. Catalases (CATs) are crucial for the defense against H_2O_2-producing insect infections and other toxins (Vivekananthan *et al,* 2022).

According to Miller *et al.,* (2004), *M. anisopliae* exhibits enhanced catalase-peroxidase activity during germination and growth, which may be required to counterbalance the hyperoxidant condition brought on by oxidative metabolism. Peroxisomal catalase has been hypothesized to play a role in the breakdown of insect hydrocarbons in this fungus (Pedrini *et al.,* 2013). Hernandez *et al.* (2010), found that the cellular differentiation, detoxification, and catabolism processes are all aided by the catalase genes found in fungi.

2.7.2. Peroxidase activity

St. Leger (1996) stated that one way that ROS can kill cells is by starting and spreading lipid peroxidation, which leads to the breakdown of cell membrane integrity and increases permeability to ions and fluids. The defense system includes antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase (Joanisse and Storey 1996). Several enzymatic activities associated with penetration, toxic effects, and pathogenicity have been identified during *M. anisopliae* host infection (Charnley, 2003).

The EPF have been shown to contain a variety of oxidative stress proteins, such as catalases, peroxidases, superoxide dismutases (SOD), and thioredoxins, as well as components of the signaling network that regulate these processes (Wang et al., 2013; Li *et al.,* 2015; Ortiz-Urquiza and Keyhani, 2015; Zhang *et al.,* 2016). As per Li *et al.* (2019), the host penetration (aspersoria formation) stage occurs when bifunctional catalase-peroxidase is essentially just increased and altered in cellular location.

2.7.3. Chitinase activity

According to St. Leger *et al.* (1986a and 1991), *M. anisopliae* has two different chitinases which are controlled and stimulated by chitin degradation product. Extracellular chitinase, lipase, and protease activity were present in measurable levels in highly pathogenic strains, and these enzymes are needed for cuticle penetration (Samuels *et al.*, 1986). As per St. Leger *et al.* (1996) in various fungi, including *M. anisopliae*, chitinases have been linked to virulence.

As per St. Leger *et al.* (1987) and Mathivanan *et al.*, (1998) the mycoparasitic and entomopathogenic fungi produce chitinases after invasion takes place. According to St. Leger (1996), chitinases play a physiological role in the development and proliferation of hyphae in fungi. Barreto and staats (2004) cited that chitinase development in *M. anisopliae*, where the enzyme manifested at high levels in response to the presence of chitin, its typical substrate, may be relevant in this case.

As per the study of St. Leger (1986), to grow on an insect's cuticle, an entomopathogenic fungus must develop a complicated mixture of chitinolytic enzymes. Chitinases play a variety of roles in an expanding number of biological systems (Gooday, 1999). The strains of *M. anisopliae* that have been studied so far are prolific chitinase producers (Screen, 2001; St. Leger, 1991).

A substantial number of plant pathogenic fungi are effectively controlled by genes encoding for fungal and plant chitinases (Bolar *et al.*, 2000). Chitin breakdown and cell wall disintegration are part of the chitinase mechanism of action, which results in fungal cell lysis (Dahiya *et al*, 2006). Additionally, chitinases may inhibit phytopathogenic activity, which would considerably lessen the farming community's reliance on harmful chemical plant protection techniques (Anwar *et al.*, 2019).

2.7.4. Protease activity

Protease is important in the early stages of invasion during penetration for *M. anisopliae* (St. Leger *et al*., 1987; 1991). St. Leger *et al.*, (1996) reported that a number of proteases have been found to be strongly expressed during fungal penetration of the host, *Manduca sexta*, and an overexpression of the protease gene (pr1) in *M. anisopliae* enhanced toxicity against *M. sexta*. The most crucial

components of the infectious process are thought to be proteases (Mustafa and Kaur, 2009).

The synthesis of cuticle-degrading enzymes by the EPF can provide as evidence of the pathogenicity of the organisms toward their hosts (Pinto *et al.*, 2002). Wang *et al.,* (2002) reported that exopeptidases and amino peptidases continued to break down the dissolved proteins into amino acids to supply EPF with food (Wang *et al.,* 2004). The cuticle of insects and mites contains up to 70% protein, fungus proteases appear to be particularly crucial to the penetration process (Charnley, 2003).

According to Bai *et al.,* (2012) and Ramanujam *et al.,* (2011), one of the most virulent entomopathogenic microbes, *M. anisopliae* was characterized as producing high amounts of extracellular enzymes (chitinase, lipase, and protease) for breaching host cuticles. Isolates with high protease activity are predicted to have high pathogenicity toward their host (Elhakim *et al,* 2020).

Production of proteases, lipases, and chitinases aids in the penetration of the host cuticle, whether the host is an insect or another type of arthropod (St. Leger *et al.*, 1996). The EPF, *M. anisopliae* breaks through the initial and most significant host defense against infection by secreting hydrolytic and lipolytic enzymes (such as proteases, chitinases, lipases, and esterases) and mechanical pressure (Silva *et al.,* 1994). Krishnaswamy (2019) found that extracellular enzymes like lipase, chitinase, and protease are secreted by *B. bassiana* to break down the main components of the insect's cuticle, allowing hyphal penetration.

2.7.5. Lipase activity

In some infection systems, lipases released by pathogenic bacteria may function as virulence factors (Stehr *et al.,* 2003). Schofield *et al.,* (2005) and Stehr *et al.,* (2004) found that numerous pathogenic bacteria have been identified that produce lipolytic enzymes, and these enzymes have been shown to play a variety of roles in the infection process. According to Stoytcheva *et al.,* (2012), lipases are a class of water-soluble enzymes that can function at the boundary between aqueous and organic phases.

Stehr *et al.,* (2003) mentioned that to facilitate the colonization of several EPF, lipolytic activity contributes to cell proliferation and hyphal development. *M.*

anisopliae adopts a synergistic method of hydrolytic enzyme release, such as proteases, chitinases, and lipases, along with mechanical processes to pierce the host cuticle (Silva *et al.,* 2005).

Stehr *et al.,* (2004) reported that the lipid molecules are found in the epicuticle, which serves as the first line of defense against arthropod pathogenic microorganisms which highlights the significance of lipolytic enzymes in the early stages of infection. In the study conducted by Silva *et al.,* (2005), it has been proven that the lipolytic activity of *M. anisopliae* strongly interacts with both the spore surface and the mycelium of the fungus. Pre-penetration growth of *M. anisopliae* on the host cuticle was correlated with lipids (Jarrold, 2007).

Maia *et al.,* (2001) and Mahadik *et al.,* (2002) studied the numerous environmental parameters including carbon sources, pH, and temperature have been extensively examined as factors that boost lipase productivity in certain fungal species. According to Ali *et al.,* (2009), the temperature and pH variations also had an impact on the ability of *M. anisopliae* to produce lipase.

Ferron (1978) also found that these enzymes contribute to infection by penetrating the host integument.Entomopathogenic fungi begin the infection process by producing lipases, chitinases, and proteolytic enzymes (Samuels and Paterson, 1995). Ali *et al.,* (2009) indicated that lipids and proteins are essential components of the insect cuticle, therefore the ability of *M. anisopliae* to produce more lipase and protease could be very advantageous for its usage as a bioinsecticide.

2.7.6. Total protein content

Protease, chitinases, and lipases are secreted by EPF in order to break down the main cuticle components and allow hyphal penetration (Wang *et al.,* 2005). Nada (2015) stated that proteins are essential parts of all living cells and contain a variety of substances like enzymes, hormones, and antibodies that are required for an organism to function properly.

As per El-banna *et al.,* (2012), insect physiological processes that depend on protein to induce ovulation and egg formation are slowed down by low protein content. Dillon and Charnley (1989) and St. Leger *et al.,* (1989) found that conidial germination of *M. anisopliae* includes protein synthesis and food absorption. Jiang *et*

al., (2019) reported that genes which are crucial for virulence in the wheat disease *Fusarium graminearum*, were found to be considerably elevated after plant infection in genome-wide loss-of-function experiments on these genes. Progenesis Same Spots software was used to identify 85 and 90 total protein spots in *M. anisopliae* (Syazwan, 2018).

According to comparative genomic research, EPF often encode fewer genes for proteins than plant pathogens, pointing to different functional processes across the fungi (Shang *et al*., 2016). Li *et al.,* (2019) and Zhang *et al.,* (2011) mentioned that in *M. oryzae*, MoRgs7 (homologous to Gprk), which is primarily engaged in appressorium development and complete virulence with cAMP signaling through the MoCrn1-dependent endocytic pathway, performs crucial functions in fungal growth and pathogenicity.

2.7.7. Trehalose content

Trehalose, the non-reducing disaccharide that serves as a trehalase substrate, has the special ability to protect membranes and enzymes from drying up and becoming inactive due to heat (Maheswari *et al.,* 2000). According to Rangel *et al.,* (2008) microorganisms that thrive in challenging conditions typically have a variety of defense systems and intracellular trehalose and mannitol accumulation is a significant process to overcome stress.

Most insects have trehalose (O-D-glucopyranosyl [1 1]-Dglucopyranoside) as their primary blood sugar and a significant source of energy for their tissues (Thompson, 2003). Mullins (1985) indicated that trehalose is the primary carbohydrate in insect hemolymph and may be significant in the natural glucose supply. Becher *et al.,* (1996) indicated that this disaccharide serves as the main sugar during flight.

As per Thompson and Dahlman (1999) trehalose concentration is crucial for controlling insect growth and development because it influences food preference and feeding behavior. Many insects, such as *Schistocerca gregaria* and *Manduca sexta*, which may be a possible nutrition source for insect pathogenic fungi like *Metarhizium anisopliae*, have trehalose as their primary hemolymph sugar. (Thompson, 2003; Sato *et al.,* 1997; Elbein, 1974). According to Zhao *et al.*, (2009), trehalose-hydrolyzing enzyme might be crucial for the pathogenesis of fungi.

Hottiger *et al.* (1987) found that heat shock and other environmental conditions, such as exposure to heavy metals, oxidants, and organic solvents, can promote trehalose accumulation. The lifespan of conidia during long-term storage has been increased due to the optimization of trehalose content (Hallsworth and Magan, 1996). According to the report of Fujii *et al*., (1996) trehalose is crucial for maintaining tolerance under particular stress conditions.

As per the findings of virgilio *et al.* (1991), trehalose must be considered as a potentially significant source of nutrients for pathogenic fungi like *M. anisopliae*, which are primarily restricted to the hemolymph, before death. Trehalose protects cells from a variety of stresses by keeping membranes intact and maintaining proteins in their natural state (Herdeiro *et al.* 2006; Singer and Lindquist 1998).

2.8. Protein profiling using SDS PAGE

Uncertainty exists regarding the molecular mechanism behind the response to heat stress in the conidia of entomopathogenic fungus. Understanding the mechanisms of thermotolerance in fungal conidia and enhancing thermal resistance depends on the definition of gene pathways linked to thermotolerance and the classification of these genes into functional categories.

2.8.1. Presence of heat shock protein in *Metarhizium anisopliae*

Heat shock proteins (Hsps) have been overexpressed in a variety of organisms to increase their tolerance to heat and other abiotic stimuli. HSPs are widely known for facilitating thermotolerance (Richter *et al.,* 2010). As per Zhao (2009), a group of conserved polypeptides known as heat shock proteins is rapidly synthesized by insects in response to high temperatures and other chemical and physical stresses (Hsps).

Xavier *et al.*, (1996) showed that HSFs from *B. bassiana, M. anisopliae, Tolypocladium nivea, P. farinosus,* and *V. lecanii* bind to the heat shock element (HSE) constitutively (non-shocked), and that heat shock produced more HSF-HSE complexes and reduced their mobility. Xu *et al.,* (2010), mentioned that Hsps can shield organisms and cells from heat harm. According to Liao *et al.* (2014), when conidia are exposed to environmental stress, the presence of HSP helps them survive.

Selkirk *et al.* (1987) demonstrated that viruses, bacteria, fungi, and insects trigger Hsp genes to provide defense against stresses. Using suppression subtractive

hybridization, high temperature can change the expression of Hsps and other genes in a vector mosquito population (Zhao *et al.*, 2009). Colinet *et al*., (2009) found that Hsps are crucial to the healing process for treating cold damage. The Hsp genes were found to be significantly up-regulated genes for anhydrobiosis, a latent state where some insects may withstand the loss of almost all of their body water content (Cornette *et al.*, 2011).

A fast rise in the production of a group of HSP family proteins can result from the disruption of regular cellular processes (Cheng *et al.* 2007). Since HSPs are upregulated in the appressoria, it is possible that their role in protein chaperoning extends beyond the acute heat shock adaptation to the maintenance of homeostasis (Liao *et al.*, 2014). St. Leger (1996) identified that a dramatic up-regulation of heat shock proteins at the early stage of *Metarhizium* contact with the insect host (appressoria development) suggested the presence of a widespread stress condition (Hsps).

The HSPs appear to have two main roles: either as chaperonins that directly mediate protein folding or as molecular chaperones that recognize and bind to nascent polypeptide chains and partially folded intermediates of proteins, preventing their aggregation and misfolding (Fink, 1999). According to Liu *et al.*, (2003), HSPs speed up protein trafficking, inhibit the buildup of protein precursors, and absorb complexes containing unfolded proteins to preserve their transport capacity.

MATERIALS AND METHODS

MATERIALS AND METHODS

The research work entitled “Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance” was carried out in the Department of Agricultural Entomology, College of Agriculture, Vellanikkara during 2020-2022. The materials used and methods exercised for the study for accomplishing the objectives are given below.

3.1. Revival of existing strains of *Metarhizium anisopliae* from the repository of department

The strains of *Metarhizium anisopliae* which had been isolated from soils and maintainted in PDA media were revived as per Kaushik and Dutta (2016). The cultureswere transferred intonew PDA media amended with the antibiotic chloramphenicol using an inoculation loop. After seven days of incubation it was again subcultured on PDA agar slants and incubated at 26 ± 2^{o}C for 10 days (Sahyaraj and Borgio, 2006). There after it was maintained under refrigerated conditions for future use.

3.2. Periodic subculturing of *Metarhizium anisopliae* isolates

The isolates were maintained by periodic subculturing on PDA plates. A fungal disc of 5mm diameter was taken using a cork borer and placed at the centerof a Petri plate containing PDA media and incubated at 26 ± 2^{o}C. The strains were purified by hyphal tip method and maintained in PDA slants and incubated at room temperature for further use (Rachappa *et al*., 2009).

3.3. Characterization of *Metarhizium anisopliae* isolates

The isolates were characterised based on both cultural and morphological parameters by growing on PDA plates and slide culture techniques.

3.3.1. Slide culture technique

Microscopic slide culture was prepared by placing filter paper or blotting paper in the lid and base plate in a petri plate and a coverslip was placed over two glass rods. The entire plate was sterilized in an autoclave and the assembly is brought inside the laminar air flow and a square-shaped piece of plain agar was placed over the slide. A loopful of spores was taken from the 10-day old culture of *Metarhizium*

anisopliae and inoculated at four corners of the plain agar. A cover slip was placed carefully over the plain agar piece without bubble formation. Then it was inoculated at room temperature. Observations were made at 48 h interval using a compound microscope and photographs were taken using computer-based software (Gebremariam, 2022).

3.3.2. Cultural characterization

The isolates were characterized based on their growth pattern on PDA plates. The diameter of the colony, colour, growth pattern, sporulation and rate of growth on the PDA plates were recorded (Gebremariam, 2022).

3.3.3. Morphological characterization

According to Bischoff *et al*., (2009) observations on morphological characters of 14-day-oldcultures were recorded using a phase contrast microscope with image analysing facility. The shape and size of spores, hyphal characteristics, and development of phialides were observed under microscopes. Photomicrographs of the isolates were taken using the software Radical.

3.4. Preservation and storage of *Metarhizium anisopliae* isolates

As per Francardi *et al*. (2016), the isolates were subcultured on PDA media and incubated for 10 days and preserved on PDA slants as well as in glycerol, and stored at 4^{o}C in a refrigerator and -80^{o}C in deep freezer. A 5mm disc of the isolates from 10 day old plates were taken using a cork borer and kept in vials with 10 per cent of glycerol in water. These vials were stored in deep freezer for long term future use.

3.5. *In- vitro* screening of *Metarhizium anisolpiae* isolates for temperature tolerance

The *M. anisopliae* strain M4 with accession number MN538359 and two *M. anisoplaie* isolates (OP97533 and OP97534) available in the Department of Agricultural Entomology were used for the study. After species-level identification, the *Metarhizium* isolates were subjected to further stress screening trials and the promising strains were used for further studies.

3.5.1. Screening on Potato Dextrose Broth (PDB)

The *Metarhizium* isolates were screened for their temperature tolerance as per the protocol of Li and Feng (2009). The isolates were inoculated on Potato Dextrose Broth (PDB) for 10 days at various temperature levels at an increasing order starting from 30to 40^{o}C. A 5mm disc was taken from 10 day old culture of isolates using a cork borer and inoculated into PDB and incubated in a BOD incubator for 10 days. The temperature tolerance was studied at temperatures of 30, 32, 34, 36, 38, and 40^{o}C using a BOD incubator.

The mycelial mat of 10 day old culture was taken out using forceps and dried on Whatmann no.1 filter paper disc for 2 h. The filter paper was made into conical shape and clipped which placed on sterilized conical flask. The mycelial mat was then taken and placed over the filter paper for 2 h in order to remove excess broth from the mat. After 2 h the fresh weight of mycelial mat was taken using a weighing balance (Acheampong, 2011).

The isolates were screened for temperature tolerance on PDA plates at increasing level of temperatures from 30^{o}C to 40^{o}C in BOD incubator as per the guidelines of Yeo *et al*., 2003. A fungal disc of 5mm was taken from 10 days old culture using a cork borer and kept at the centre of PDA plate and incubated for 10 days. Radial growth of the colony was measured after 10 days. The plates were turned upside down and diameter of the surface growth was measure using a ruler (Acheampong, 2011).

3.6. Screening on PDB amended with Poly Ethylene Glycol (PEG)

The isolates were screened for drought tolerance by growing on PDB amended with PEG (6000). In order to create a drought condition in this media, polyethylene glycol (PEG) 6000 was added (PEG-adjusted media) to the PDB (Humphreys *et. al*, 1989). The isolates were subjected to increasing level of drought condition by adding suitable amount of PEG 6000 to PDB. Isolates were incubated on PEG amended PDB at 10, 20, 25, 27.5, 30, 32.5, 34, 36, 38, 39 and 40 per cent. A disc of 5mm diameter was taken from 10 day old culture plate and inoculated into the PEG adjusted PDB and incubated for 10 days at room temperature. The fresh weight of mycelial mat after 10 days was measured and spore characters were studied by generating photomicrographs on computer software.

3.7. Inducing temperature tolerance in *Metarhizium anisopliae* isolates

After screening, the selected isolates CKD, EKM2 and M4 (OP97533 and OP97534 and MN538359) were subjected to increasing levels of temperature to induce temperature tolerance. Preparation of medium and inoculation was done by the procedure as explained in 3.5.1. The isolates were incubated at increasing levels of temperature from 35^{o}C to 42^{o}C. The strains which had grown at one level of temperature was inoculated to next incremental level of temperature. A loopful of spore was taken from 10 day old culture grown at 35^{o}C and inoculated to PDB which was then incubated at 36^{o}C in BOD incubator. The procedure was repeated till 42^{o}C temperature was reached. The measurements were taken according to the procedure mentioned in 3.5.2.

3.7.1. Potato Dextrose Agar (PDA) plates

A 5mm disc was taken from the PDA plate grown at 35^{o}C and inoculated in the centre of PDA plate and incubated at 36^{o}C in BOD incubator. The process was repeated upto 42^{o}C temperature. Measurements were taken according to the procedure mentioned in 3.5.3.

3.7.2. Growing and maintaining selected thermo tolerant isolates for six generations

A loopful of spores from the culture grown at the highest temperature was taken and inoculated into fresh PD broth andincubated again at the same temperature. Repeated subculturing were carried out six times in order to confirm its thermo tolerance.

3.8. Inducing drought tolerance in *Metarhizium anisopliae* isolates

The drought induction was carried out by addingPolyethylene Glycol (PEG) to Potato Dextrose Broth (PDB) as mentioned in 3.6.1. The isolates were subjected to increasing levels of concentrations of PEG from 25 to 41 per cent. The experiment was started at a PEG concentration of 25 per cent and continued up to 35 per cent as broad range screening, followed by 37, 38, 39, 40, and 41 per cent. Isolates grown in one level of PEG concentration were subjected to the next incremental level of PEG concentration and incubated at room temperature (26 ± 2^{o}C) for 10 days. After 10 days of inoculation, the mycelial weight of the colony was measured. The mycelial

mat was taken using forceps and dried in Whatman No.1 filter paper for 2 h. After 2 h of drying, the fresh weight of mycelia was taken in a weighing balance.

3.8.2. Growing and maintaining the selected thermo tolerant isolate for six generations

A loopful of spores from the selected drought tolerant isolate was taken and inoculated into fresh PD broth andgrown again at the same temperature for ten days. This was repeated up to 6 generations of the selected drought tolerant isolate in order to confirm its drought tolerance.

3.9. *In vitro* screening of *Metarhizium anisopliae* for bio control efficacy

The selected tolerant strains were tested for their biocontrol efficacy on laboratory host, the greater wax moth (*Galleria mellonella*) according to the protocol of Gebremariam (2022).

3.9.1. Preparing spore suspension

Conidia were collected from the culture surface under aseptic conditions by saturating the plate with sterile distilled water and a surgical scalpel is used to remove the colony. Two to three drops of 0.1% Tween 80 was added to the solution for thorough mixing and mixed well using a blender. The solution was filtered through sterilized muslin cloth. Using an enhanced Neubauer Hemocytometer, the conidial count in each suspension werecalculated and spore count was adjusted to10^9 (Lomer and Lomer, 1996).

$$\text{No. of spores/ml} = \frac{D \times X}{N \times K}$$

where, D = Dilution factor

X = No. of spores counted from the smaller square

N = No. of squares in the smaller square

$K = 2.5 \times 10^{-7}$

The conidial suspension of each isolates were then serially diluted to lower concentrations of 10^5, 10^6, 10^7 and10^8 spores ml^{-1}.

3.9.2. Rearing of greater wax moth (*Galleria mellonella*)

Wax moth larvae were collected from Banana Research Station (BRS), Kannara. The larvae were reared on semi synthetic diet containing wheat flour (350g), corn flour (200g), milk powder (130g), baking yeast powder (70g), honey (100ml) and glycerol (150ml) in plastic containers at constant temperature of 26 ± 2^{o}C. Fifth instar larvae were used for bioassay (Ibrahim *et al*., 2016).

3.9.3. Bioassay of *Metarhizium anisopliae* isolates on wax moth (*Galleria mellonella)* larvae

According to Kavallieratos *et al*. (2014), *G. mellonella* larvae were placed in petri dishes without feeding and sprayed with fungal conidial solutions. The larvae pre starved for twoh in separate plastic boxes were used for bioassay.Each Petri dish received 10 number of larvae. The larvae were then placed in sterile Petri plates with sterile tissue paper, and respective spore suspensions of isolates (1 ml) were evenly sprayed on top of the larvae. The experiment was carried out with three replications, and a control was maintained by spraying sterile water. Observations on mortality were made at 24 h intervals and continued up to 10 days, and the per cent mortality was determined using Abbott's formula after the appropriate adjustments (Abbott, 1925). To estimate the dose-mortality relationship, the LT_{50} (Lethal time required to kill 50% of test populations), the LT_{90}, and fiducial limits probit analysis were performed using the Polo Plus software.

3.10. Biochemical analysis of stress tolerance and virulence

In order to confirm the stress tolerance and to know the mechanisms involved, the selected *M. anisopliae* isolates were analyzed biochemically. The selected temperature tolerant *metarhizium* isolate (EKM2, OP97534), drought tolerant isolate (CKD, OP97533) and the susceptible isolate (M4, MN538359) were subjected to biochemical analysis. Catalase and peroxidase, the two antioxidant enzymes that increase tolerance to various stress were tested. The chitinase activity was estimated to determine the chitin degrading ability. Trehalose, a disaccharide that provides energy and aids in survival, when exposed to freezing and dehydration was also measured. The activity of the hydrolytic enzyme, lipase and the cuticle degrading enzyme, protease and total protein content were also quantified in order to analyze the stress tolerance.

3.10.1. Sample preparation for total protein and enzyme assay

For total protein, catalase and peroxidase assay, the fungal extracts were prepared as described by Sujatha and Padmaja (2014). Selected tolerant strains EKM2 (OP97534) and CKD (OP97533) after drought and temperature induction were maintained in PDB at 26 ± 2^{o}C. Mycelial mats taken from 10 day old culture was washed using sterile water and stored in refrigerator for biochemical analysis.

Fungal mycelial mats of 4.5 g each were weighed and each isolate was pulverised in a mortar and pestle with 10 ml of sodium phosphate buffer (pH 7.4) for the estimation of total protein. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C after being sonicated for 8 min. Until estimation, the supernatant produced was kept in a deep freezer (-18^{o}C).For estimating catalase and peroxidase, 4.5 g of the mycelial mat from each isolate was ground in a precooled pestle and mortar with 10 ml of ice-cold sodium phosphate buffer (pH 7). It was then centrifuged at 18,000 rpm for 15 min for the peroxidase test and 10,000 rpm for 10 minat 4°C for the catalase assay after being sonicated for 8 to 10 min. The supernatant was used within 24 h period.

3.10.1.1. Preperation of standard bovine serum albumin (BSA) solution and reagents for total protein assay

The total protein assay was carried out according to the standard protocol of Lovrein and Matulis, (1995). Bovine serum albumin was used to make a stock solution by mixing 50 ml of distilled water with 50 mg of BSA. Working standard solution was prepared from this stock solution by pipetting out 10 ml of stock solution and making upto 50 ml with distilled water. This means that every 1 ml of the solution contains 200 µg protein. After that, different aliquots of 200 µl, 400 µl, 600 µl, 800 µl and 1000 µl were pipetted into different test tubes and volume was made up to 1 ml using distilled water. A blank with distilled water alone was also kept. Four reagents *viz*. Reagent A, B, C, and D were prepared as follows.

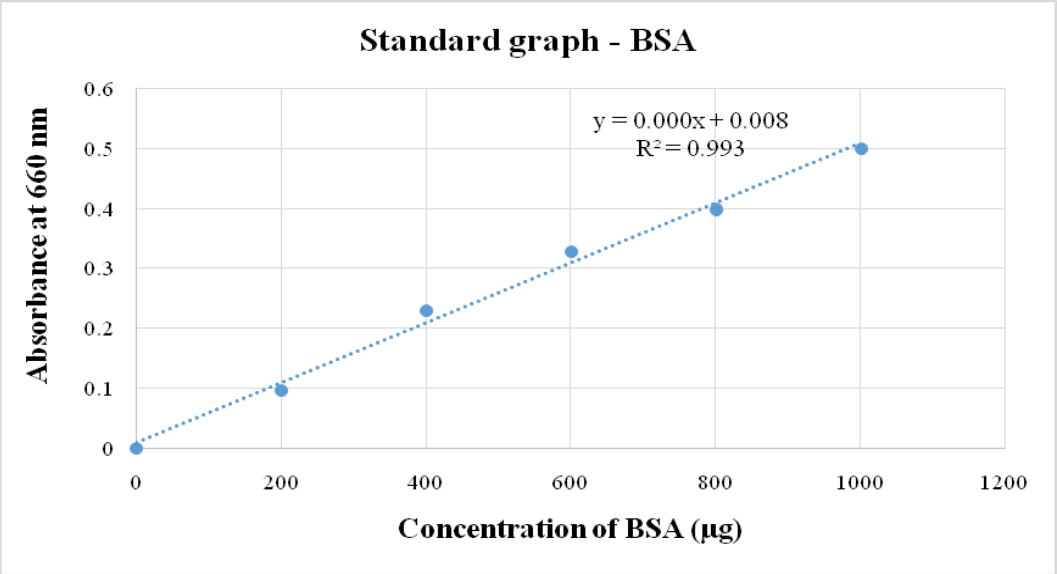
Sl. No.	Reagents	Preparation
1	Reagent A	2% Sodium carbonate dissolved in 0.1 N NaOH
2	Reagent B	0.5% Copper sulfate solution dissolved in per cent sodium potassium tartrate solution
3	Reagent C	Mixture of freshly prepared reagent A (50 ml) and reagent B (1 ml)
4	Reagent D	Freshly prepared Folin- ciocalteu reagent and distilled water taken in 1:1 ratio

3.10.1.2. Preparation of standard graph using BSA solution

Reagent C (5ml) was added to all test tubes, including the blank ones. Then, each aliquot was well mixed and held for 10 min. 0.5 ml of reagent D was added after 10 min, and it was then kept at room temperature for 30 min in the dark. Using an Agilent Cary 60 UV Vis spectrophotometer, an absorbance reading at 660 nm was taken when the contents turned blue. A standard graph was created using the measured OD value and the BSA concentrations. Using the standard graph, protein content was calculated and represented in mg/ml.

3.10.1.3. Total protein assay of selected *Metarhizium anisopliae* isolates

Sample preparation of selected *M. anisopliae* isolates were carried out asdescribed in 3.10.1. A test tube containing 50 µl of supernatant was added to 2.5 ml of reagent C, which was then thoroughly mixed. After mixing, the mixture was incubated for 10 min and 0.25 ml of reagent D was added to it, and was kept at room temperature for 30 min in the dark. Agilent Cary 60 UV Vis spectrophotometer was used to measure the absorbance at 660 nm.



3.10.2. Catalase activity of selected *Metarhizium anisopliae* isolates

Catalase activity was estimated as per the standard protocol of Hadwan, (2016) with some modifications.For estimating the catalase activity, hydrogen peroxide buffer was prepared by adding 0.16 ml of H_2O_2 into 100 ml sodiumphosphate buffer (p^H7). Three ml of H_2O_2-phosphate buffer was added to 50 µl of supernatant and mixed well with a glass rod in a cuvette. Supernatant mixed with H_2O_2 -free phosphate buffer was kept as control. Using an Agilent Cary 60 UV/Vis spectrophotometer, absorbance at 240 nm was measured at every 30 s for 5 min. Catalase activity was expressed in terms of change in absorbance per minute per mg of protein.

3.10.3. Peroxidase activity of selected *Metarhizium anisopliae* isolates

The peroxidase activity of the isolates was assessed as per the protocol of Mahadevan and Sridhar (1986). A clean, dry cuvette containing two millilitres of sodium phosphate buffer, one millilitre of 20 mM guaiacol, and 50µl of supernatant was placed in an Agilent Cary 60 UV/Vis spectrophotometer. Finally, 50µl of hydrogen peroxide (10 mM) were added to the cuvette, and the absorbance was immediately measured at 470 nm for five min at 30 seconds intervals. The peroxidase activity was determined using the observed change in absorbance per minute. It was calculated as a change in absorbance per minute divided by tissue weight.

Peroxidase activity =

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

3.10.4. Lipase activity of selected *Metarhizium anisopliae* isolates

The lipase activity was measured as per the titrimetric determination method of Pinsirodom and Parkin (2001). In this process, triacylglycerols, the natural substrates, are hydrolyzed to produce fatty acids. At regular intervals, subsamples were taken from reactive mixtures, and ethanol was added to quench the reactivity. By directly titrating with NaOH to a thymolphthalein end point, fatty acid releaseof the reaction was quantified.

Sl. No.	Reagents	Preparation
1	95% ethanol	95 ml of 100 per cent ethanol made up to 100 ml using distilled water
2	1% thymolphthalein indicator	1 g thymolphthalein indicator made up to 100 ml using distilled water
3	Olive oil/gum arabic emulsion substrate	Add 10 ml olive oil and 10 g of gum arabic in a 400 ml beaker and bring the volume to 200 ml with 50 mM sodium phosphate buffer (pH 8) and homogenized for 5 min using a domestic blender without formation excessive foam
4	Sample for analysis (0.5 ml)	Crush the mycelial mat in a pestle and mortar using 10 ml of sodium phosphate buffer (pH 8) and centrifuge at 10000 rpm for 15 min at 4 ^{o}C and the supernatant was taken and stored at 4 ^{o}C.
5	0.05 N NaOH	Sodium hydroxide pellets 2g were taken and made up to 1000 ml using distilled water
6	Sodium phosphate buffer (pH 8)	5.3 ml of monobasic sodium phosphate buffer and 94.7 ml of dibasic sodium phosphate buffer were mixed

3.10.4.1. Preparation of reagents

3.10.4.2. Estimation of lipase activity of *Metarhizium anisopliae* isolates

Six 25 ml Erlenmeyer flasks were taken and to that add 10 ml of 95 per cent (v/v) ethanol and 2 to 3 drops of 1 percent thymolphthalein indicator which serve as the titration cocktail were added.A 50 ml Erlenmeyer flask was takento which 50 ml of 5 percent olive oil/gum arabic emulsion was poured to the substrate and it was incubated for 15 min at 37°C in a water bath with magnetic stirring. After that 0.5 ml of test sample was added to to initiate lipolysis on the emulsion substrate and continued stirring.

At five suitable reaction intervals of 5, 10, 15, 20, and 25 min, 5 ml reaction mixture was removed and subsample were transferred to flasks containing titration cocktail prepared earlier. Swirl contents immediately to stop the reaction. The contents of each flask were titrated with 0.05 N NaOH using a burette until a light blue colour appears. To the Erlenmeyer flask containing titration cocktail add olive oil/gum arabic emulsionand 0.5 ml of phosphate buffer was added and mix well. Using a burette the contents of this flask were titrated with 0.05 N NaOH to serve as blank.

3.10.4.3. Calculation

The quantity of fatty acids liberated in each subsample was calculated based on the equivalents of NaOH used to reach the titration end point using the following equation:

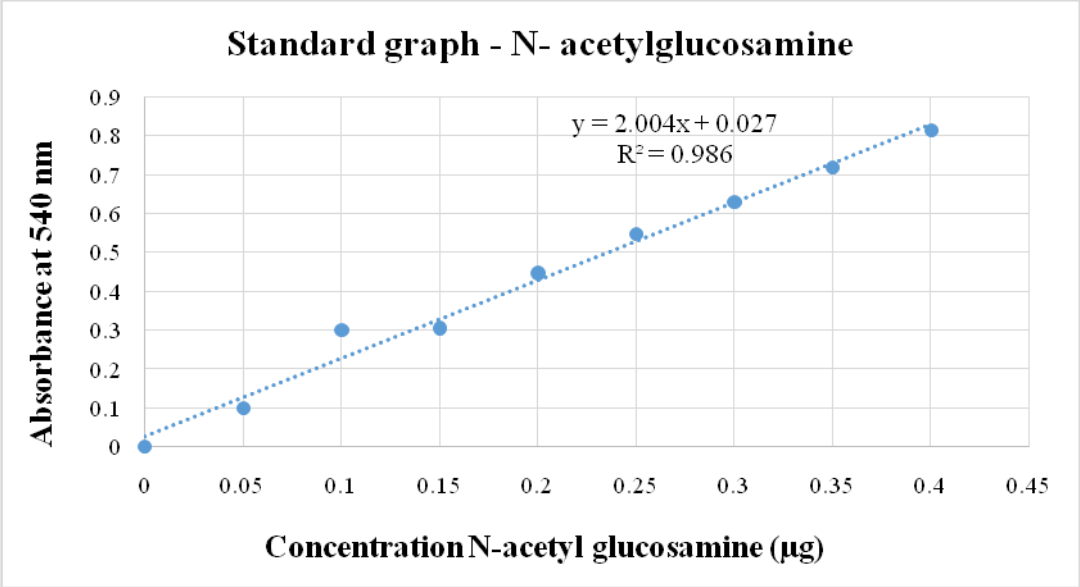
µmol fatty acid/ml subsample = [(ml NaOH for sample - ml NaOH for blank) × N × 1000]/5 ml

3.10.5. Chitinase activity

The chtinase activity was measured using Dinitrosalicylic Acid (DNS) Assay as per the procedure of Kankanamge (2017). To totally rule out other carbon and nitrogen sources and to determine whether chitin can be employed as the only carbon and nitrogen source, chitin and water liquid media was also used for the DNS test. Chitin was the only carbon source in the media, which should release chitobiose and N – acetyl glucosamine upon putative chitinase activity by the fungus.

3.10.5.1. Preparation of the standard graph using N-acetyl glucosamine

N-acetyl glucosamine stock (20mg/ml) was used to make series of dilutions of N- acetyl glucosamine. From this stock solutions, standard solutions of 0.05 µl /ml, 0.1µl /ml, 0.15µl /ml, 0.2µl/ml, 0.25µl /ml, 0.3µl/ml were prepared then to 0.5ml of each dilution, 0.5ml of DNS was added. Then it was heated at 95ºC for 10 min and absorbance was measured at 540nm. A graph for absorbance at 540nm (Y axis) against respective concentration of N- Acetyl glucosamine (X axis) was plotted.



3.10.5.2. Colloidal chitin preparation

The colloidal chitin preparation was carried out by following the standard protocol of Roberts and Selitrennikoff (1988). Forty gram chitin was kept at 4ºC and 250 ml ice cold concentrated hydrochloric acid was added very slowly with continuous stirring for one hour. Glass wool was used to filter the mixture and it was then collected into 2L ice cold water under stirring with magnetic stirrer. The filtrate was a gelatinous white material and was separated using whatman no.1 filter paper. The obtained colloidal chitin was then repeatedly washed with tap water until it gained neutral p^H and was stored at 4ºC for further use.

3.10.5.3. Dinitrosalicylic acid assay of *Metarhizium anisoplaie* isolates

Each isolate of *M. anisoplaie* was cultured in 250ml flasks with 60µg/ml streptomycin and 60µg/ml chloramphenicol in 15ml of sterile water containing 1.5 per cent colloidal chitin.

The cultures were incubated in a shaking incubator at 100rpm at 25ºC for seven days. From that 1.5ml of each sample (treatment and control) was taken daily and were put into separate 1.5ml eppendorf tubes. These tubes were centrifuged at 9000 rpm for 30 seconds to get rid of any debris including chitin and mycelial parts. From these tubes, 1ml of the supernatant was taken into test tubes and 1ml of DNS reagent was added after that, then the tubes were heated at 95ºC for 10 min. Then tubes were allowed to cool down to room temperature. The absorbance was measured at 540 nm using UV vis Spectrophotometer. Culture with fungus grown in water alone without chitin was used as blank. The absorbance of samples was compared with the standard curve and the chitinase activity was estimated.

3.10.6. Protease activity of selected *Metarhizium anisopliae* isolates

Protease activity was carried out by following the standard protocol given by Creative enzymes, a division of creative biomart. The quantity of enzyme needed to hydrolyse casein into 1 g of tyrosine in 1 minute at a specific pH and temperature is known as an activity unit and protease activity is expressed in activity unit (U).

3.10.6.1. Preparation of standard graph using L- Tyrosine

1. Preparation of standard solution of tyrosine

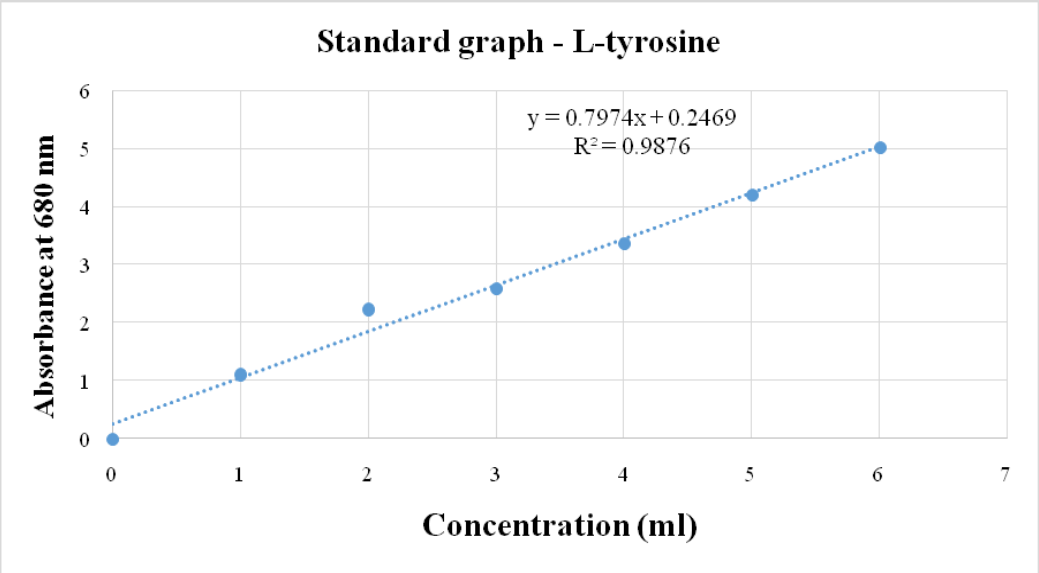
Weighed1 g L-tyrosine and dissolved in 60 mL HCl (1 mol/l). The solution was transferred into a 100 ml volumetric flask and made upto 100 ml using the HCl solution (1 mol/l). To form a solution of L-tyrosine of 1 mg/ml. After that, 10 ml of L-tyrosine solution (1 mg/ml) was made upto 100 ml with HCl solution (0.1 mol/l) in a volumetric flask to give the standard solution of L-tyrosine (100 µg/ml).

3.10.6.2. Preparation of Reagnets

	Reagents	Preparation
1	Folin's Reagent, working solution	One volume of the stock solution was combined with two volumes of distilled water to create the working solution
2	Sodium carbonate solution	Anhydrous Na_2CO_3, 42.4g was taken and dissolved in 800 mL water and made up to 1000 ml in a volumetric flask using distilled water
3	Trichloroacetic acid solution (TCA)	Trichloroacetic acid, 65.4 g was dissolved in 800 mL water and the solution was made up to 1000 ml in a volumetric flask using distilled water
4	Sodium hydroxide solution	Sodium hydroxide pellet, 20 g was added into 900 mL DI water and the solution was stirred until dissolving and made up to 1000 ml in a volumetric flask using distilled water
5	Hydrochloric acid solution (1mol/L)	8.3 ml of HCl dissolved in 100 ml distilled water
7	Phosphate buffer solution (pH 7.5)	Weigh 27.8 g monobasic sodium phosphate and dissolve in 1000 ml water. Weigh 53.65 g dibasic sodium phosphate and dissolve in 1000 ml water. Mix up 16 ml of monobasic sodium phosphate and 84 ml dibasic sodium phosphate to obtain pH of 7.5
8	Casein solution	Casein, 1 g is mixed with 2-5 drops of the sodium hydroxide solution in a beaker. 80 ml sodium phosphate buffer (7.5 pH) solution was added into the beaker and the mixture was stirred and boiled for 30 min. The solution was then cooled down to room temperature and transferred into a 100 ml volumetric flask and made up to 100 ml using the buffer solution. It was then adjusted to the same pH as the buffer solution. The solution was then stored for 3 days at 4 °C

2. Standard curve

Working solutions were prepared using tyrosine standard stock solution. Take 0 ml, 1 ml, 2ml 3 ml, 4 ml and 5 ml in a test tube and was made up to 100 ml using distilled water. One ml of each was taken and to that 5 ml sodium carbonate solution and 1 ml working solution of Folin's reagent was added. The test tubes were then incubated at 40 ± 0.2 °C in a water bath for 20 min. The absorbance of each solution was then measured in a 10 mm cuvette at 680 nm. The standard curve was generated using linear or parabolic regression, with absorbance as the response in the y axis and the final tyrosine concentration as the variable in the x axis.



3.10.6.3. Estimation of protease activity of *Metarhizium anisopliae* isolates

Sample preparation

Crushed fungal sampleof 1-2 g was taken and dissolved with the buffer solution. The solution should be further diluted to 10-15 ml with the buffer solution. The casein solution solution was incubated in a water bath at 40 ± 0.2 °C for 5 min.

The test sample of 1 ml was incubated at 40 ± 0.2 °C for 2 min and 1 ml casein solution was added to that, mixed well and incubated at room temperature for 10 min. After that 2 ml of trichloroacetic acid solution was added, mixed well and incubated at 40 ± 0.2 °C for 10 min. Solution was then filtered using Whatman No. 1 paper. The mixture of 1 mlwas taken and to that 5 ml of sodium carbonate solution and 1 ml

working solution of Folin's reagent were added and incubated at 40 ± 0.2 °C for 20 min. The absorbance was measured at 680 nm in a 10 mm cuvette. The solution without sample content served as blank.

3.11.6.3. Calculation

The protease activity was calculated using the following equation. The activity X is in U/ml or U/g.

$$X = \frac{A \times V1 \times V2 \times n}{M \times t}$$

A: The reading from the standard curve at the given Absorbance of 680 nm

V: Total volume of the diluted sample solution in ml

V2: Total volume of the reaction mixture in ml (4 ml)

n: Dilution factor of the test sample

m: Weight of the protease sample in g

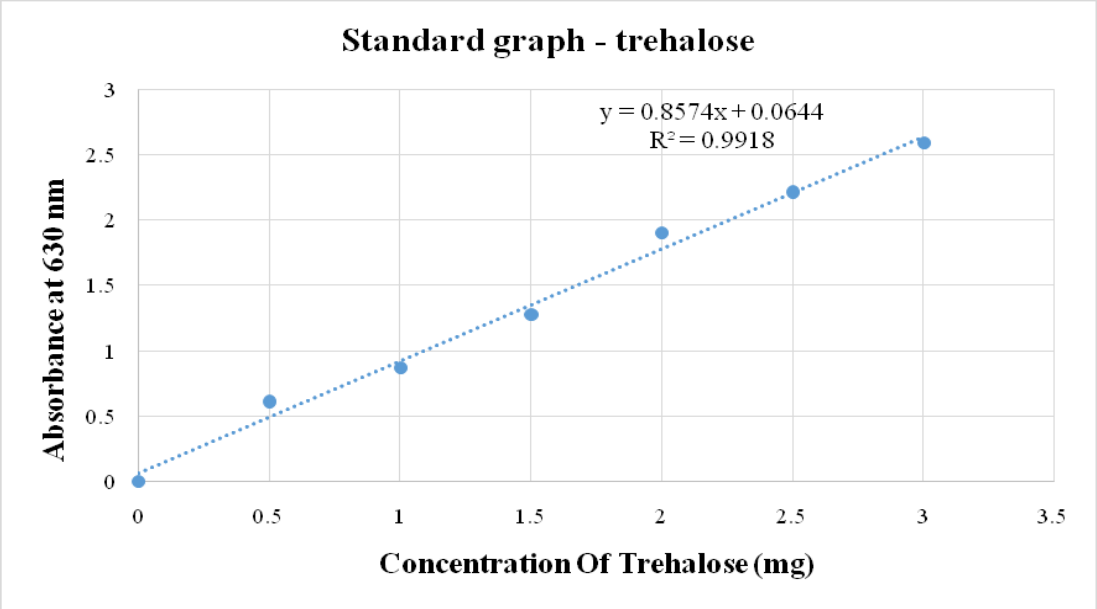
t: Reaction time in min (10min).

3.10.7. Estimation of trehalose content

The amount of trehalose of the stress tolerant isolates was determined using the anthrone-sulphuric acid colourimetric technique Wang *et al*. (1997).

3.10.7.1. Preparation of standard graph

1g of trehalose was dissolved in 1000 ml of distilled water, and six portions of the stock solution, measuring 0.5, 1, 1.5, 2, and 2.5 ml each, were pipetted into the test tubes. To each test tube containing aliquots, 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 and kept in a boiling water bath and allowed react for 10 min. Using an Agilent Cary 60 UV Vis spectrophotometer, the absorbance was measured at 630 nm after the combination had been allowed to cool at room temperature. Readingscollected corresponding to the concentrations of trehalose were used to generate a standard graph.



3.10.7.2. Preparing sample for trehalose assay

The mycelial mat for estimation was prepared according to the procedure described in 3.7.2. A mortar and pestle was used to grind about 1 g of mycelial mat and 3 ml of broth. The supernatant was centrifuged at 5000 rpm for 5 min using ice cold sterile distilled water. This process was repeated twice. After adding 4 ml of trichloroacetic acid to the produced pellet, it was centrifuged for 20 min at 10,000 rpm, and the supernatant was collected in a 15 ml centrifuge tube. The reaction was carried out three times, and the preserved supernatant was collected and diluted with trichloroacetic acid to a volume of 50 ml.

3.10.7.3. Trehalose content estimation in selected tolerant strains

An aliquot of 0.5 ml of enzyme extract from the mycelial extract prepared as previously described was combined with 5 ml of anthrone sulphuric acid and 4 ml of trichloroacetic acid. In a water bath, the mixture was heated, cooled, and the absorbance measured as previously mentioned. Trehalose content of the mycelia was calculated from the standard graph and expressed in milligram of trehalose per min per gram of mycelia.

3.11. Protein profiling using Sodium Dodecylsulfate – Poly acrylamide Gel Electrophoresis (SDS PAGE)

The protein profiling of the selected tolerant *M. anisopliae* isolates was done to identify the presence of heat shock proteins. The experiment was done according to the standard protocol of Sadasivam and Manickam (2015).

3.11.1. Fungal culture preparation

The selected isolates were grown for 14 days at 27^{o}C and the mycelial mat of the isolates are used for analysis.

3.11.2. Preparation of samples for protein isolation

The 14 day old mycelial mat was taken and ground well using a precooled mortar and pestle after adding 10 ml of sodium phosphate buffer (pH 7.4). The well ground mycelial mat was then centrifuged at 10000 rpm for 15 min at 4^{o}C. The supernatant obtained was filtered using a whatman No.1 filetr paper to remove debris and spores. The protein content of the sample is estimated using a nano drop spectrophotometer and the sample volume was made accordingly. The sample protein was added with treatemt buffer and dye (5 ml dye: 15 ml sample) and boiled in water bath at 100^{o}C for 15 min.

3.11.3. Gel casting

The gel plates were washed and wiped with tissue paper dipped in alcohol. The casting unit was prepared and the master plate (10.5 X 10.5) and base plate (10.5 X 10.5) were assempled carefully into it. The space in between the plates were filled with distilled water and kept for 5 min for checking leakage. After that the water was removed and wiped off with a tissue paper. Resolving gel (10 %) was prepared and poured into the plates immediately, upto a height just below the level of combs without forming air bubbles. A layer of distilled water was added above the resolving gel inorder to break the bubbles. The plates with resolving gel was kept as such for 30 min and the added water was decanted and wiped with tissue paper. Then stacking gel (4 %) was prepared and poured into the plates immediately above the resolving gel layer upto the top and a well comb was inserted at the top of stacking gel and allowed to polymerize the gel for 30 min.

3.11.4. Sample loading and running the gel

The casted gel was taken and the combs were removed carefully to create wells. The wells were cleaned with distilled water and the excess water was wiped off with a tissue paper. The gel plates were mounted on to the electrophoretic apparatus and fixed inside the electrophoresis tank. The electrophoretic tank was then filled upto three- fourth level using the electrode buffer. The samples were prepared as mentioned in 3.11.2 and was loaded carefully into the wells along with marker protein (Prestained protein ladder – 4 µl).

The electrophoretic unit was closed and connected to the power supply unit with care. A voltage of 60 V and current of 15 m A was set initially and then increased to 100 V and 35 m A. After the dye enters into the resolving gel, the unit was run until the tracking dye reaches three- fourth of the gel and the supply was stopped after that. The plates were carefully removed and separated. The gel was removed with care from the plates and transferred into staining box after rinsing with distilled water.

3.11.5. Silver staining of the resolving gel

The separated gel was washed with distilled water and dipped in fixing solution for 1 hour in a staining box. The fixer solution was changed and gel was kept in it for 14 h. The fixer solution was drained and then the gel was then washed in 30 % ethanol for 10 sec and repeated two times. Then it was washed with deionised water. The gel was then dipped in pretreatment solution for 1 minute and again washed with deionized water for 30 sec and repeated thrice. The gel is then impregnated in silver nitrate (0.2%) for 20 min with uniform shaking followed by washing with deionized water.It was then dipped in developing solution for 5-10 miutes until the appearance of clear bands. The gel was immediately transferred to stop solution for 30 min followed by washing with deionized water.

3.13. Statistical Analysis

Data was analysed using the KAU GRAPES (Genral R- shiny based Analysis Platform Empowered by Statistics) software. The analysis of experiments was done using two factor factorial method and tabulated based on grouping of data.

RESULTS

4. RESULTS

The study "Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance" was conducted at the College of Agriculture, Vellanikkara for developing novel strains of *Metarhizium anisopliae* through induction of temperature and drought tolerance and to evaluate the biocontrol efficacy against wax moth larvae (*Galleria mellonella*). The results of the experiment are depicted in this chapter.

4.1. Reviving of existing isolates of *Metarhizium anisopliae*

Three isolates of *M. anisopliae* being maintained in the Department of Agricultural Entomology were re-isolated from old cultures for the study. The isolate CKD and EKM2 were collected from Trissur and Ernakulam district and the isolate M4 was obtained from Alappuzha district. Serial dilution and pour plating method were employed to obtain CKD, EKM and M4 isolates and were obtained from 10^{-3} dilutions. Single spore isolation method from streaked plates was done to recover CKD and EKM. The isolate, M4 was recovered by inoculation of the mycelial disc from old culture plates into new PDA plates.

4.2. Periodic subculturing of *Metarhizium anisopliae* isolates (CKD, EKM2 and M4)

All the three isolates of *M. anisoplaie viz*., CKD, EKM2 and M4 showed full growth on the PDA plates on 14^{th} day after inoculation when stored at room temperature, 28±2 ^{o}C. The growth rate was faster in CKD and EKM2 when compared to M4. The isolates, CKD and EKM2 attained full growth on the 12^{th} day which was faster compared to M4 which showed complete coverage of the PDA plate only on the 14^{th} day. The isolates CKD and EKM2 sub cultured on PDA plates formed healthy viable colonies with high sporulation, while M4 has shown good vegetative growth with low sporulation.

4.4. Characterization of *Metarhizium anisopliae* isolates

The cultural, morphological, and molecular characteristics of the three isolates were carried out during the study. Based on these characters the isolates would be easily distinguished (Plate 1).

4.4.1. Cultural characterization of *Metarhizium anisopliae* isolates

a) CKD

The isolate was obtained from Chalakkudy of the Thrissur district. The isolate showed complete growth in the PDA plate by the 12th day. The colony was white in colour initially and gradually changed to dark green in colour with a greenish-white coloured border. It often showed circular wavy markings in the culture plate (Plate 1). The isolate showed a good amount of sporulation which was dark green in colour from 6th day onwards. The colony is a pale white colour on the reverse side of the plate (Table 1).

b) EKM2

The isolate was collected from the Eranakulam district. The isolate showed full growth on the PDA plate by the 11th day. The colony was yellowish-green in colour. Initially, the colony was dirty white in colour, later changed to pale yellowish green, and finally became dark yellowish green with dark green spores. The isolates showed abundant spores from 8th day onwards but sporulation was less when compared to the isolate CKD (Plate 1). The reverse side of the plate showed dark creamy white colour (Table 1).

C) M4

The isolate was obtained from Moncombu, Alappuzha district. The colony showed pure white fluffy growth and covered the PDA plate completely on 14th day. The white colour changed to yellow and then to light green to olive colour after 14days. The sporulation was very low compared to CKD and EKM2. The isolate has white-coloured spores from the 11th day of inoculation but in sparse amount (Plate 1).The reverse side of the plate showed a cream colour (Table 1).

4.4.2. Morphological characterization of *Metarhizium anisopliae* isolates

Morphological characters of the *M. anisopliae* isolates, CKD, EKM2 and M4 are presented in Table1.

a) CKD

The spores are greenish-coloured with an elongated dumbbell shape. Conidia are formed as clusters at the tip of phialides. The hyphae are branched and phialides

are short thick and multiple-branched clusters of conidia. The mean length and width of conidia are 7.71 µm and 1.40 µm respectively. The mean length of phialide is 13.14 µm with 4.72 as the mean width. The mean mycelial thickness observed was 6.47 µm (Plate 1).

b) EKM 2

The conidia were yellow coloured with visible septation at both ends and the shape of conidia was elongated to oval and broad in the middle. The conidia were formed as clusters at the tip of phialides. The phialides were with broader base and narrow tips. The conidia recorded a mean length of 8.33 µm and width of 2.41 µm. The mean length and width of the phialides were 9.33 µm and 3.18 µm, respectively. The mean hyphal thickness observed was 7.29 µm (Plate 1).

c) M4

The conidia were whitish in colour, long and oval with thick margins. The mean conidial length observed was 6.97 µm with a 1.34 µm width. The conidia were formed as clusters and chains at the tip of phialides. The phialides were long with uniform thickness from bottom to end. The mean length and width of phialides were 6.82 µm and 2.02 µm, respectively. The mean mycelial thickness observed was 5.12 µm (Plate 1).

Table 1. Cultural and morphological characteristics of *Metarhizium anisopliae* isolates

Sl. No.	*M. anisopliae* isolates	Cultural characters	Colour of conidia	Size of conidia (µm)	Arrangement of conidia	Size of phialides
1	**CKD**	White coloured mycelia gradually changes to dark green with dark green sporulation	Dark green colour	*7.71X 1.40	Formed as clusters at the tip of phialides	13.14X4.72
2	**EKM2**	Yellowish green coloured mycelia with dark green coloured spores	Dark green to yellowish green	8.33X2.41	Formed clusters at the tip of phialides	9.33X3.18
3	**M4**	Whitish coloured mycelia with fluffy growth with white coloured spores	White coloured	6.97X1.34	Formed both clusters and chains at the tip of phialides	6.82X2.02

*****Mean of three observations

4.4.3. Molecular characterization of *Metarhizium anisopliae* isolates

The molecular characterization of *M. anisopliae* was carried out through ITS sequencing at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The nucleotide homology of each isolate was obtained by blasting in the online BLASTn program of NCBI.

a) Sequence comparison of the isolate CKD

The nucleotide sequence captured from NCBI showed more than 99 per cent similarity with 99 per cent query coverage to *M. anisopliae* isolates, HN26Z01, SC36A02 and isolate SC50C05. The accession number of CKD obtained from the Bankit was OP97533.

b) Sequence comparison of the isolate EKM2

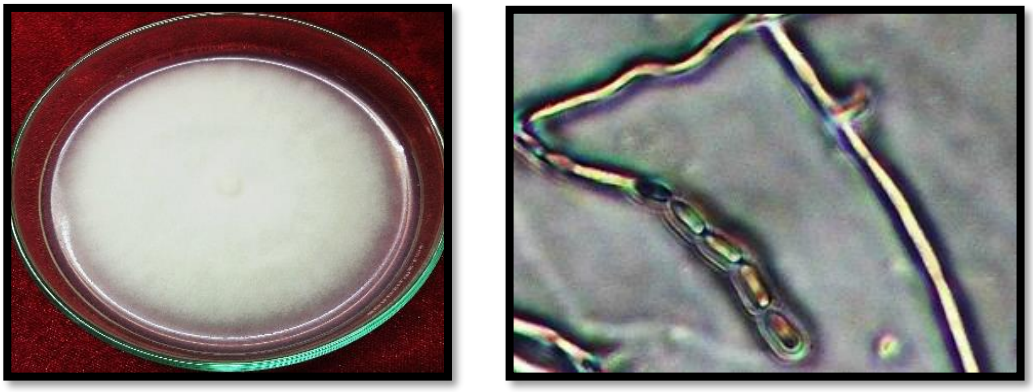
The nucleotide captured from NCBI compared to the isolate EKM2 showed 99. 58 per cent similarity with 100 per cent query cover to the *M. anisopliae* isolates MaGD46, HA02B01, HE15A01, HE19B02 and HE05B02. The accession number obtained from the Bankit was OP597534.

a) Sequence comparison of the isolate M4

The nucleotide captured from NCBI when compared to the isolate M4 showed 99.79 per cent identity with 96 per centper cent query coverage to *M. anisopliae* isolates BUM1900, M9, IIHR isolate and M-63. The accession number obtained from the Bankit was MN538359.

CKD

EKM2



M4

Plate 1. Morphological characters *of Metarhizium anisopliae* isolates

Table 2. Sequences of *Metarhizium anisopliae* isolates

Isolate	Genomic sequences
CKD	AGAACATCACTCCCACCCCTGTGATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAA CCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGG TTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT TGAACGCACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTG GGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGG CCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTT ATAGTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAAAAAGCGGGAGGAA
EKM2	CACTCCCACCCCTGTGATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTG AATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC GCACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGACTT GGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCC TCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAAACCCCCAACTTTTTATAGT TGACCTCGAATCAGGTAGGA
M4	ATTCGAGGTCACTATAAAAAGTTGGGGGGTTTTACGGCAGTGGACCGCGCCGGGCTCCTGTTGCGAGTGCTTTACTA CTGCGCAGAGGAGGGCCACGGCGAGACCGCCAATTAATTTAAGGGACGGCTGTGCTGGAAAACCAGCCTCGCCGA TCCCCAACACCAAGTCCACAGGGGACTTGAGGGGCGTAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGAC GGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTT CTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTTTTTAACCACTCAGAAGATACTTAT TAAAAAATTCAGAAGGTTTGGGTCCCCGGCGGGCGCGAAGTCCCGCCGAAGCAACAATTAAAGGTATGATCACAG GGGTTGGGAGTGGTTCTTCCGTTTTC

Table 3. Sequence homology of *Metarhizium anisopliae* in BLAST analysis

***M. anisopliae* isolates**	**Description**	**Maximum score**	**Query Coverage (%)**	**E Value**	**Identity**
CKD	*Metarhizium anisopliae* strain HN26Z01	952	99	0	99.43
	Metarhizium anisopliae strain SC36A02	946	99	0	99.43
	Metarhizium anisopliae strain HE06A01	946	98	0	99.61
	Metarhizium anisopliae strain GX02A02	941	98	0	99.42
EKM2	*Metarhizium anisopliae* strain MaGD46	876	99	0	99.58
	Metarhizium anisopliae strain HA02B01	876	99	0	99.58
	Metarhizium anisopliae strain HE19BO2	876	99	0	99.58
	Metarhizium anisopliae strain HE15A01	876	99	0	99.58
M4	*Metarhizium anisopliae* strain BUM1900	859	96	0	100
	Metarhizium anisopliae strain M9	859	96	0	100
	Metarhizium anisopliae strain IIHR	859	96	0	100
	Metarhizium anisopliae strain M-36	859	96	0	100

4.5. Screening of *Metarhizium anisolpiae* isolates for temperature tolerance

Screening of the *M. anisolpiae* isolates was done at different temperature levels starting from 30^{o}C to 40^{o}C. The weight of mycelia and radial growth of the colony were recorded at all temperature levels.

4.5.1. Screening of *Metarhizium anisolpiae* isolates for thermotolerance on Potato Dextrose Broth, (PDB)

The screening of *M. anisopliae* isolates was done in the Potato Dextrose Broth (PDB) at different temperature levels starting from 30^{o}C. The results of the experiment are depicted in the Table 4.

At 30^{o}C, the isolate CKD showed the highest mycelial weight (5.122g) followed by EKM2 (4.221g). The lowest mycelial weight was shown by the isolate M4 (0.606) which is significantly low compared to the other isolates (Plate 2). At 32^{o}C, EKM2 showed the highest mycelial weight (7.353g) followed by CKD (5.394g). The isolate M4 had shown the lowest mycelial weight (2.382 g) (Plate 3). When the temperature was increased to 34^{o}C, EKM has again shown higher mycelial biomass followed by CKD and least growth was observed in the isolate M4 (Plate 4). The isolate, EKM2 showed highest weight of mycelia (7.338g) at an enhanced temperature level of 36^{o}C and it was followed by CKD and M4 (Plate 5). The highest mycelial weight at a temperature of 38^{o}C was observed in the isolate EKM 2 (6.221g) followed by CKD (0.897g) which is significantly lower than that showed by EKM2. The mycelial weight of M4 at 38^{o}C was the lowest among the three isolates (0.328g) (Plate 6). None of the isolates showed growth at 40^{o}C (Plate 8).

From the thermal screening experiment, it is observed that at all temperature levels tested, the isolate EKM2 has shown the highest mycelial weight which was significantly superior when compared to the isolates CKD and M4. Isolate CKD had shown significantly higher mycelia weight at all temperature levels compared to M4. The isolate M4 had shown only sparse growth at all temperature levels when compared to CKD and EKM2. The mycelial weight of EKM2 ranged from 4.221g to 9.971g which has proved to be superior to other isolates. The isolate M4 with mycelial weight ranging from 0.328g to 0.606 g was found to be susceptible to temperatures when compared with the other two isolates *ie*. EKM2 and CKD.

The sporulation of the isolates at all treatment levels was also observed. The highest sporulation was shown by CKD followed by EKM2, while isolate M4 recorded very low sporulation at all temperature levels. At 30^{o}C, CKD has showed highest sporulation followed by EKM2 and least sporulation was observed in the M4 isolate. The sporulation was observed to be gradually reducing with increasing temperature levels. At the highest temperature level of 38^{o}C, sporulation was highest

in CKD followed by a moderate sporulation in EKM2. The isolate M4 recorded least sporulation at higher temperature levels with no sporulation at 38ºC (Plate 7).

Among the three isolates, EKM2 recorded highest mycelial weight and moderate sporulation and was found to be temperature tolerant and it was followed by CKD with better mycelial weight and highest sporulation. The isolate M4 with lower mycelial weight and least sporulation at all the levels of temperatures and was considered as a susceptible isolate to temperature.

Table 4. Effect of temperature on the mycelial weight *of Metarhizium anisopliae* isolates

Mean mycelial weight at different temperature level (g/100ml)						
Isolates	**Control (28±2ºC)**	**30ºC**	**32ºC**	**34ºC**	**36ºC**	**38ºC**
CKD	5.501^{d}	5.122^{de}	5.394^{de}	3.359^{fg}	5.699^{d}	0.897^{h}
EKM2	5.250^{a}	6.243^{ef}	7.353^{c}	9.971^{b}	7.338^{c}	6.221^{cd}
M4	3.703^{h}	2.241^{ij}	2.382^{g}	0.317^{h}	3.663^{f}	0.328^{h}

*Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

Table 5. Effect of temperature on sporulation *of Metarhizium anisopliae* isolates

Isolates	**Sporulation at different temperature levels**				
	30ºC	**32ºC**	**34ºC**	**36ºC**	**38ºC**
CKD	+++	+++	+++	+++	+++
EKM2	+++	+++	+++	+++	+++
M4	+	+	+	+	–

+++: High sporulation, ++: Medium sporulation, +: Sparse sporulation, -: No sporulation

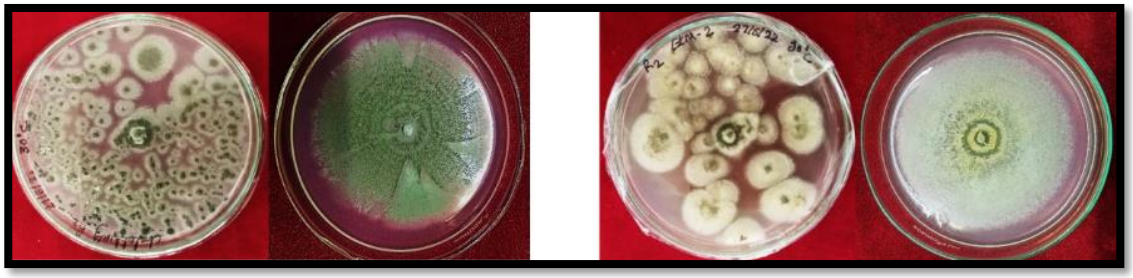
4.5.1. Screening of *Metarhizium anisolpiae* isolates for thermotolerance on Potato Dextrose Agar (PDA) Plates

The screening of the *M. anisopliae* isolates at different temperatures was done in the PDA plates and the radial growth of the colony was measured. The per cent

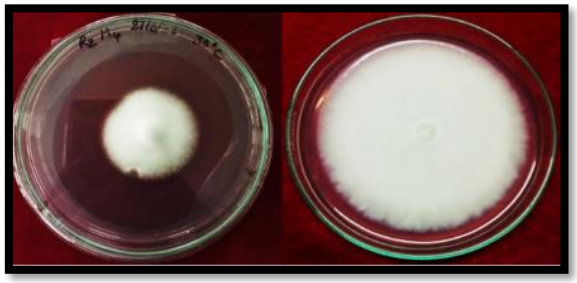
CKD CONTROL

EKM2 CONTROL

M4 CONTROL



CKD EKM2



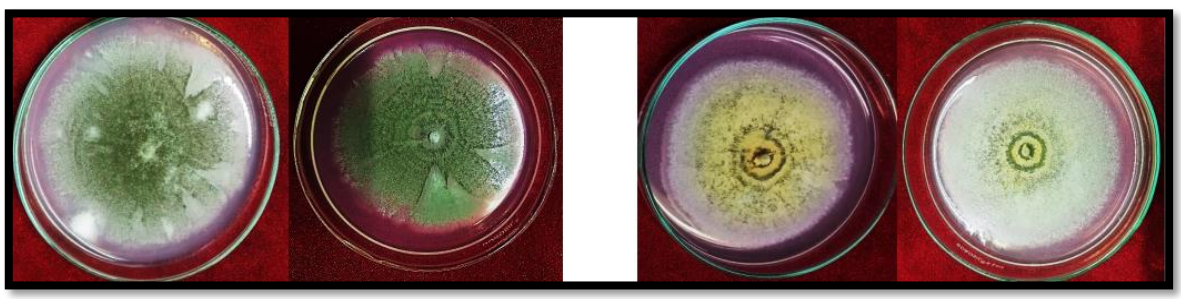
M4

Plate 2. Growth and sporulation of *Metarhizium anisopliae* isolates at 30°C

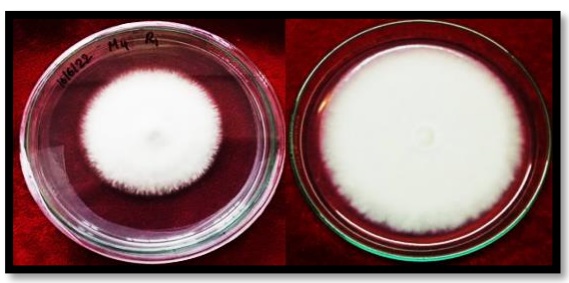
CKD CONTROL

EKM 2 CONTROL

M4 CONTROL



CKD EKM2



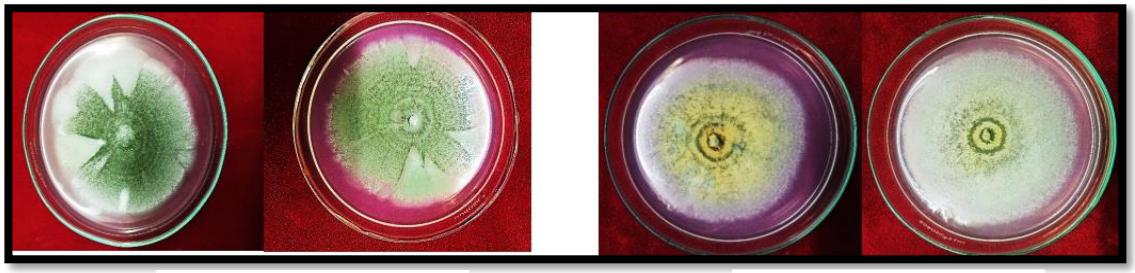
M4

Plate 3. Growth and sporulation of *Metarhizium anisopliae* isolates at 32ºC

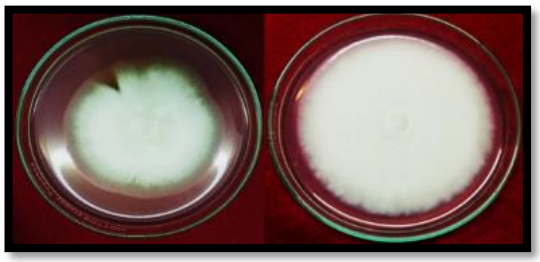
CKD CONTROL

EKM2 CONTROL

EKM2 CONTROL

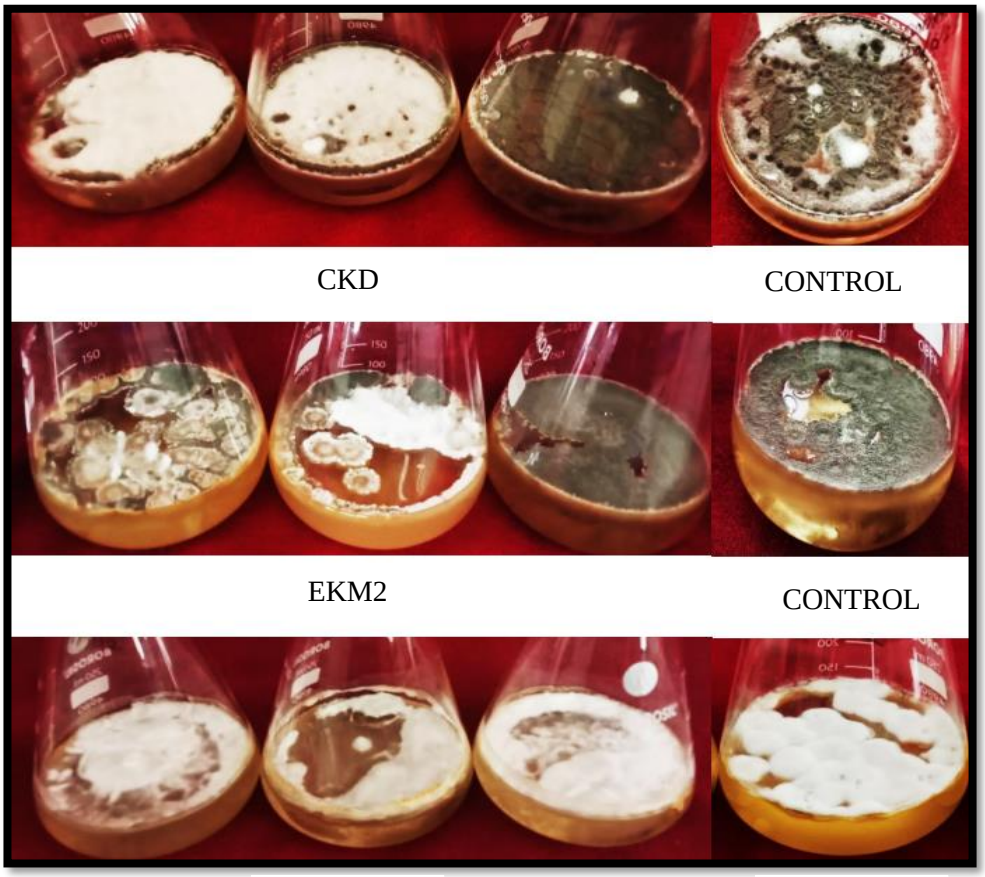


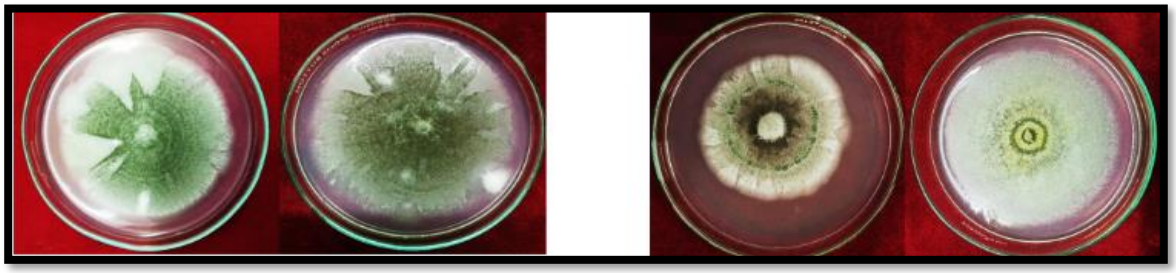
CKD EKM2



M4

Plate 4. Growth and sporulation of *Metarhizium anisopliae* isolates at 34ºC





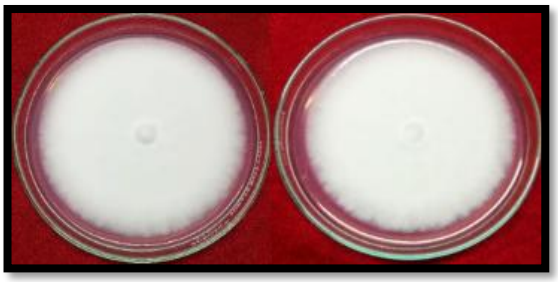


Plate 5. Growth and sporulation of *Metarhizium anisopliae* isolates at 36°C

growth inhibition was recorded in order to estimate the temperature tolerance. The results of the experiment are shown in the Table 5.

At 30^{o}C, the lowest level of growth inhibition was shown by CKD (52.77%) and highest level of inhibition was shown by the isolate M4 (69.62%). The isolate, EKM2 has also shown a growth inhibition of 55.55 per cent which is higher than CKD, but significantly lower than M4 (Plate 2). When the temperature was increased to 32^{o}C, the lowest inhibition percentage was shown by EKM2 (49.99%), followed by CKD (53.70 %), whereas M4 has shown the highest percentage inhibition (78.88%) which is significantly higher than the other two isolates (Plate 3). At 34^{o}C, both CKD (60.18%) and EKM2 (60.18%) has shown lowest inhibition percentage when compared to M4 and were on par with each other. The isolate, M4 showed highest inhibition percentage at 34^{o}C (78.883%) (Plate 4).

When the temperature was enhanced to 36^{o}C, the highest inhibition percentage was shown by M4 (75.55%), and lowest was shown by both CKD (60.18%) and EKM2 (61.10%) which were on par with each other (Plate 5). At an increased temperature of 38^{o}C, highest inhibition was shown by EKM2 and M4 which were on par with each other (77.03 and 77.77 % respectively) and the lowest was observed in the isolate CKD (Plate 6). None of the isolates has showed growth at 40^{o}C (Plate 8). At all the temperature levels tested, the highest percentage inhibition was recorded in the isolate M4. The isolate EKM2 has shown least percentage inhibition which indicates that EKM2 is superior to other isolates. The isolate M4 showed highest percentage inhibition indicating that, M4 is least tolerant and susceptible to thermal stress.

Table 6. Effect of temperature on inhibition of *Metarhizium anisopliae* isolates

Inhibition of *Metarhizium anisopliae* isolates (%)					
Isolates	**30^{o}C**	**32^{o}C**	**34^{o}C**	**36^{o}C**	**38^{o}C**
CKD	52.773^{gh}	53.700^{gh}	60.183^{f}	60.183^{f}	72.220^{de}
EKM2	55.550^{g}	49.997^{h}	60.183^{f}	61.107^{f}	77.030^{c}
M4	69.627^{e}	78.883^{c}	78.883^{e}	75.550^{cd}	77.770^{c}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

The sporulation of the isolates in the PDA plates at all treatment levels was observed. The highest sporulation was showed by CKD followed by EKM2. The isolate M4 showed very low sporulation at all temperature levels. At 30^{o}C, CKD has showed highest sporulation followed by EKM2 and least sporulation was observed in M4 isolate. The sporulation was observed to be gradually reducing with increasing temperature levels. At the highest temperature level of 38^{o}C, sporulation was highest in CKD, followed by a moderate sporulation in EKM2. The isolate M4 has shown least sporulation at higher temperature levels with no sporulation at 38 ^{o}C (Plate 7).

Table 7. Effect of temperature on sporulation *of Metarhizium anisopliae* isolates

Isolate	Sporulation at different temperature levels				
	30^{o}C	32^{o}C	34^{o}C	36^{o}C	38^{o}C
CKD	+++	+++	+++	+++	+++
EKM2	+++	+++	+++	+++	+++
M4	+	+	+	-	-

+++: High sporulation, ++: Medium sporulation, +: Sparse sporulation, -: No sporulation

4.6 Screening of *Metarhizium anisolpiae* isolates for drought tolerance

Screening for drought tolerance was conducted at different concentrations of polyethylene glycol (PEG) in PDB. The PDB amended with different concentrations of PEG was used for the experiment.

Table 8. Effect of drought on growth of *Metarhizium anisipliae* isolates

Mean mycelial weight at different concentrations of Polyethylene Glycol (PEG)(g/100ml)											
Isolate	**Control**	**10%**	**20%**	**25%**	**27.5%**	**30%**	**32.5%**	**34%**	**36%**	**38%**	**39%**
CKD	3.913^{b}	2.434^{jklm}	2.140^{mn}	2.619^{ijk}	2.868^{ghi}	2.967^{efgh}	3.034^{efg}	2.950^{fgh}	3.157^{defg}	2.587^{ijk}	2.172^{lmn}
EKM2	6.732^{a}	3.005^{efg}	3.117^{efg}	3.445^{cd}	3.554^{c}	3.276^{c}	3.027^{efg}	3.185^{def}	3.235^{def}	2.323^{klmn}	2.055^{n}
M4	2.956^{fgh}	1.101^{p}	0.372^{r}	0.448^{qr}	1.617^{o}	0.715^{q}	2.483^{jkl}	2.667^{hij}	2.117^{n}	1.467^{o}	1.336^{op}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

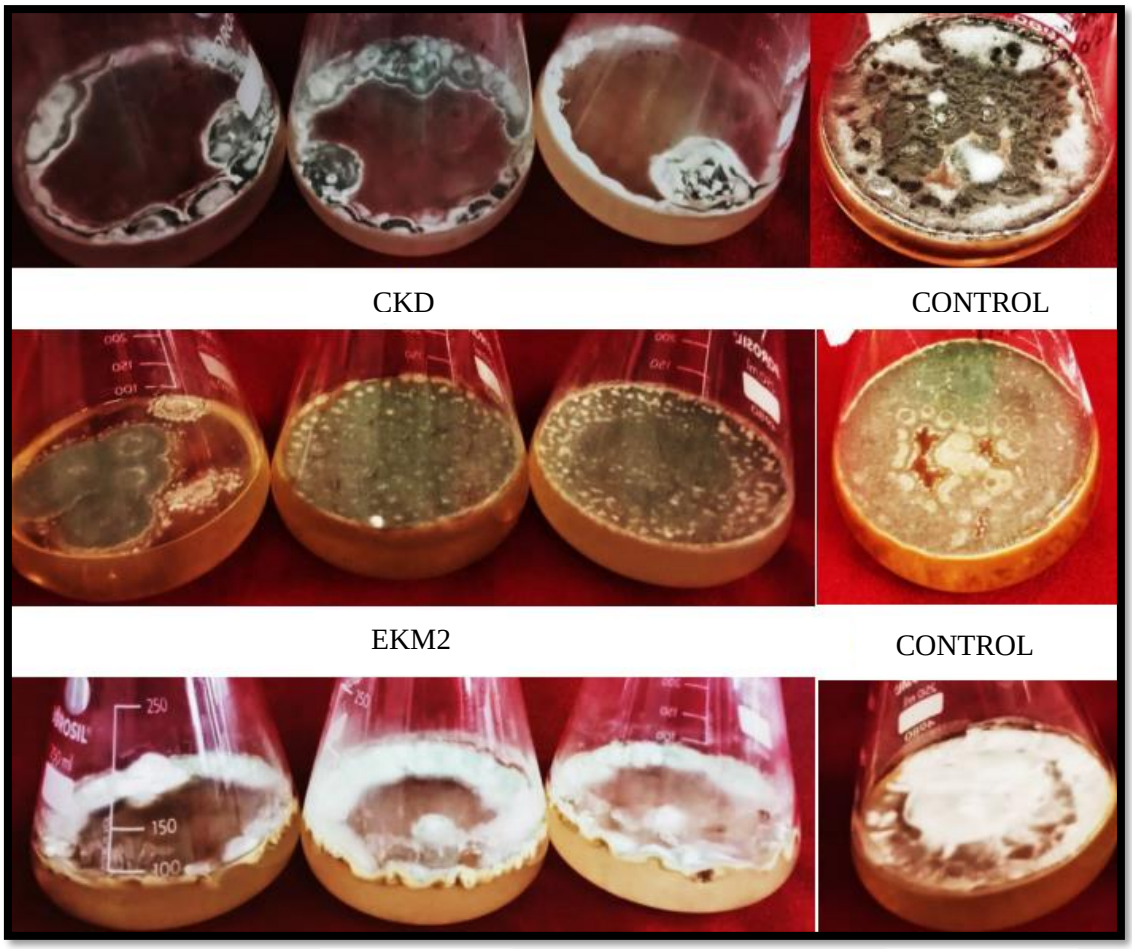
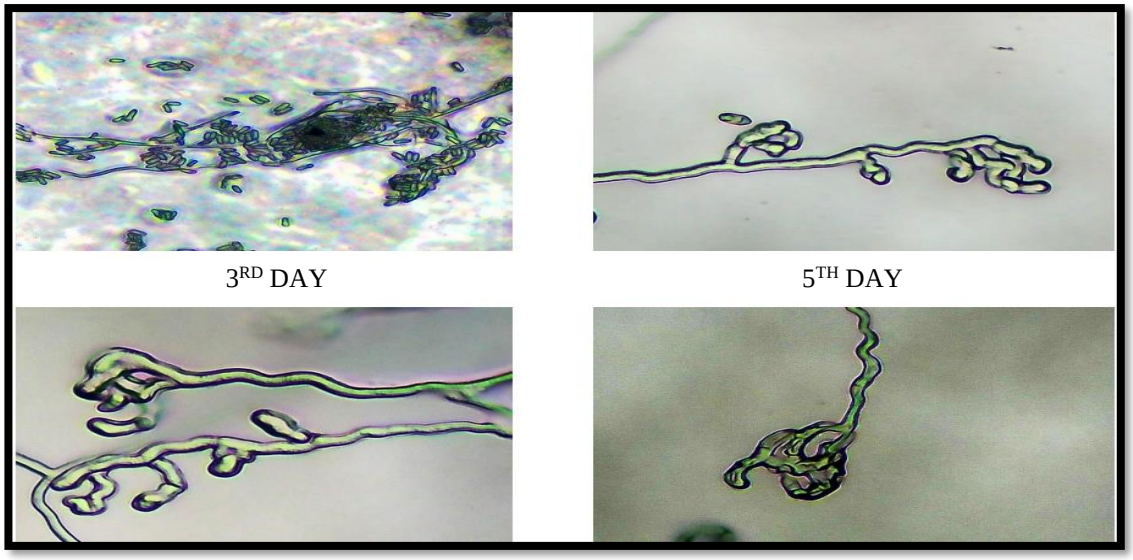
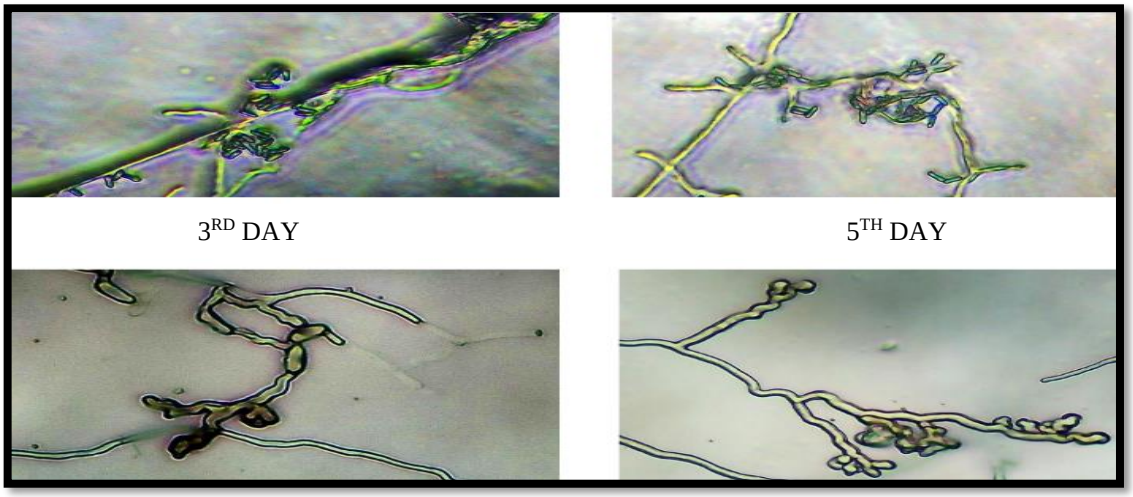


Plate 6. Growth and sporulation of *Metarhizium anisopliae* isolates at 38ºC



3RD DAY 5TH DAY

7TH DAY 9TH DAY



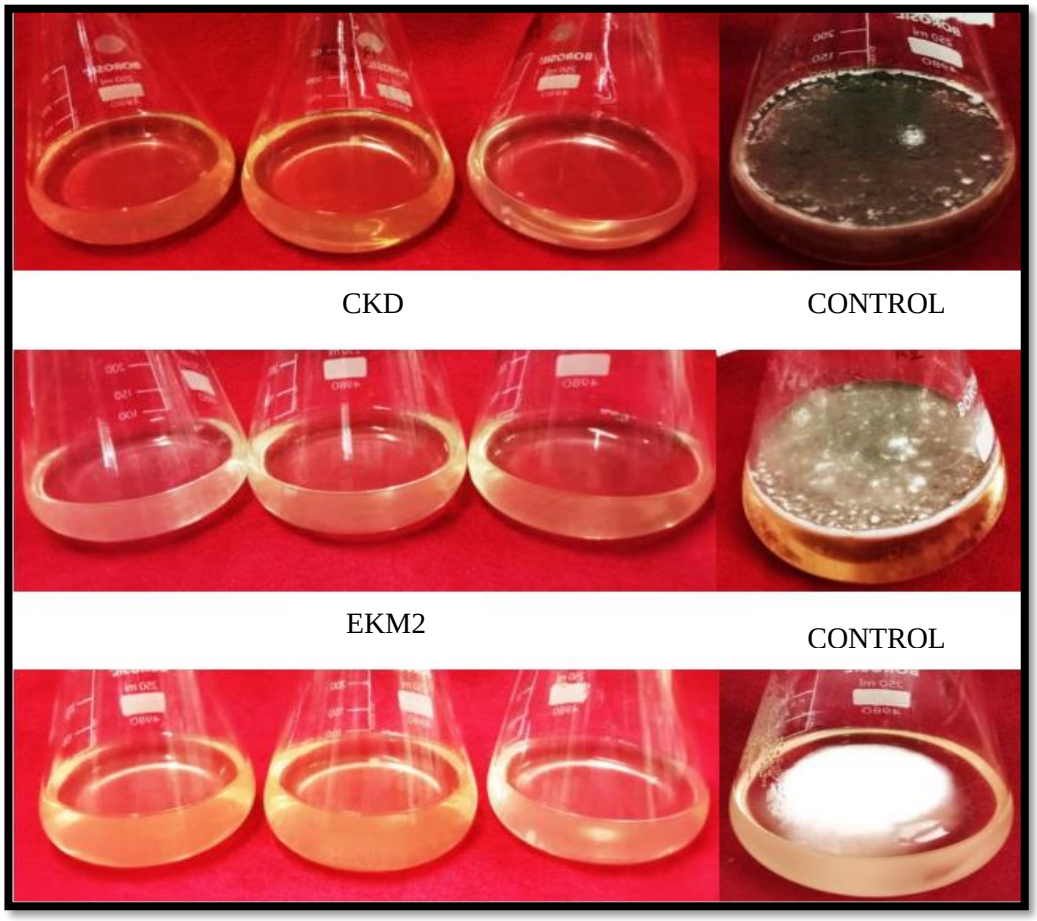
3RD DAY 5TH DAY

7TH DAY 9TH DAY

3RD DAY 5TH DAY

7TH DAY 9TH DAY

Plate 7. Sporulation and hyphal growth of *Metarhizium anisopliae* isolates at 38°C



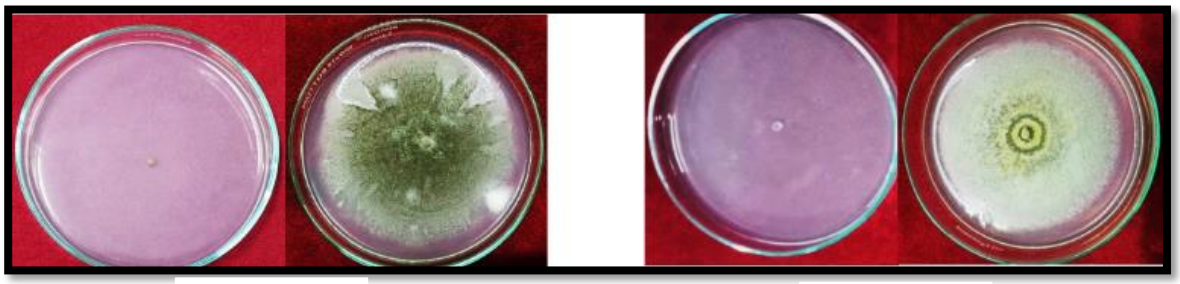


Plate 8. Growth and sporulation of *Metarhizium anisopliae* isolates at 40ºC

Drought experiments are conducted initially with PEG starting from 10 % concentration and weight of mycelia was recorded. The results of the experiment are given in the table 6.

At the lowest concentration of PEG, (10%) the isolate EKM2 has shown the highest mycelial weight (3.005g) followed by CKD (2.434g). The least growth was observed in the isolate M4 (1.10g) which was significantly lower compared to the other isolates (Plate 9). At 20% of PEG the highest mycelial weight was recorded in the isolate EKM2 followed by CKD and the lowest growth was observed on the isolate M4 (Plate 9). The same trend was observed when the PEG concentrations were increased to 25, 27.5, 30, 32.5, 34, and 36 percentage (Plate 10). At all these concentrations of PEG, isolate EKM2 showed higher biomass ranging between 3.02g to 3.55 g followed by CKD which ranged between 2.172g to 3.157g. The isolate M4 showed sparse growth with lowest mycelial weight ranging between 0.37g to 2.11g. When the PEG concentration was increased to 38%, the trend was changed and CKD recorded the highest mycelial weight (2.58g) followed by EKM2 (2.32g). The isolate M4, was showing lowest mycelial weight (1.46g) at 38% also (Plate 11). At the peak PEG concentration of 39%, CKD has recorded the highest biomass (2.17g) followed by EKM2 (2.05g). M4 was the one which has recorded the lowest mycelial weight (1.33g). The highest concentration of PEG at which growth was observed was 39% (Plate 11). At 40% of PEG none of the isolate has shown growth. At the highest PEG concentration CKD has shown higher biomass, hence it has proved to be superior to other isolate. M4 has shown least weight and proved to be susceptible to drought. While EKM2 has shown moderate growth.

The sporulation of the isolates were recorded and the isolate CKD has shown highest sporulation at all levels of PEG concentration. The isolate EKM 2 has shown moderate sporulation at all the PEG concentrations. M4 was with least sporulation at all levels of PEG concentration (Plate 12).

From the experiment it is found that isolate CKD has shown higher biomass with higher sporulation at the highest PEG concentration. Hence CKD was proved to be drought tolerant and the isolate M4 with lowest mycelial weight and least sporulation was identified as susceptible. EKM 2 was moderately tolerant with moderate sporulation.

Table 9. Effect of drought on sporulation *of Metarhizium anisopliae* isolates

Isolate	Sporulation at different concentrations of PEG									
	10 %	20%	25%	27.5%	30%	32.5%	34%	36%	38%	39%
CKD	+	++	+++	+++	+++	+++	++	++	++	+
EKM2	+	++	+++	+++	+++	++	++	++	+	_
M4	-	+	+	+	+	-	-	-	-	-

+++: High sporulation, ++: Medium sporulation, +: Sparse sporulation, -: No sporulation

4.7. Inducing temperature tolerance in *Metarhizium anisopliae* isolates

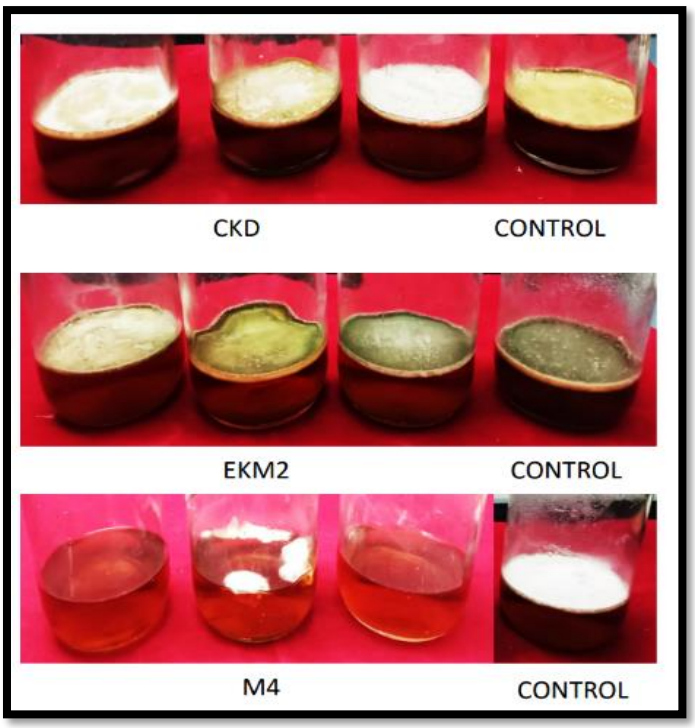
Inducing temperature tolerance to *M. anisopliae* isolates was done at increasing levels of temperature starting from 35^{o}C and continued upto 42^{o}C and the experiment was done as explained in 3.5. The mycelial weight of the isolates, radial growth of the colony, size and shape of the spores, mycelial thickness, spore germination and spore count at each temperature level was recorded. The results of the experiment are depicted below.

4.7.2. Effect of induced temperature on mycelial weight of *Metarhizium anisopliae* isolates

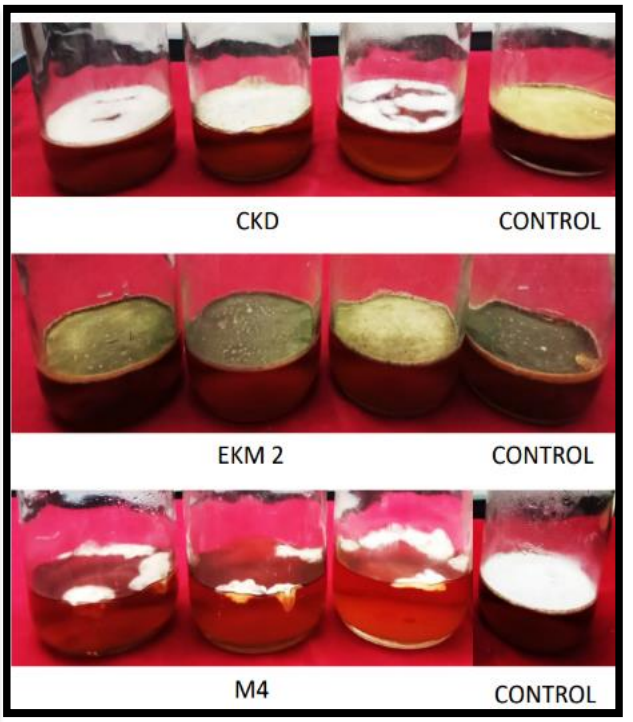
The isolates were subjected to different temperature levels starting from 35^{o}C, continuously in order to impart temperature tolerance.

4.7.2.1. Effect of induced temperature on mycelial weight of *Metarhizium anisopliae* isolates on potato dextrose broth

The mycelial weight of the colony after ten days of incubation in the PD broth were recorded. The results of the experiment are depicted in the Table10. At the lowest temperature level of 35^{o}C, highest mycelial weight was recorded in the isolate EKM2 (12.47g/100 ml) followed by CKD (4.83g). The lowest weight of the mycelial growth was shown by the isolate M4 (4.21g) (Plate 12). The mycelial weight of EKM 2 was significantly higher compared to the other isolates. At 36^{o}C, EKM2 has shown a mycelial weight of 12.75g followed by CKD and M4. The isolates CKD and M4 has shown weights which are on par with each other and was significantly lower when compared to the isolate EKM2 (Plate 14). When the isolates were grown at a higher temperature of 37^{o}C also, the highest mycelial biomass was observed in the isolate



10%



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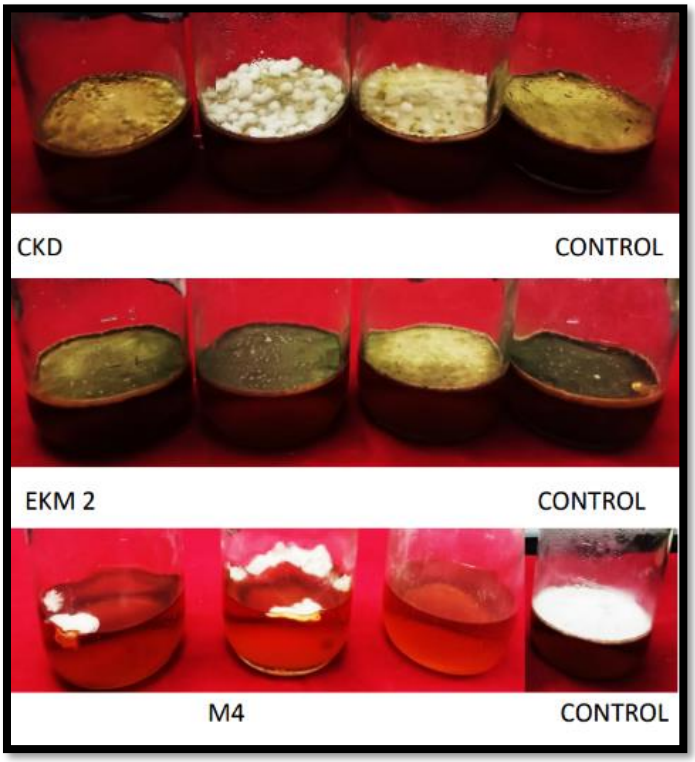
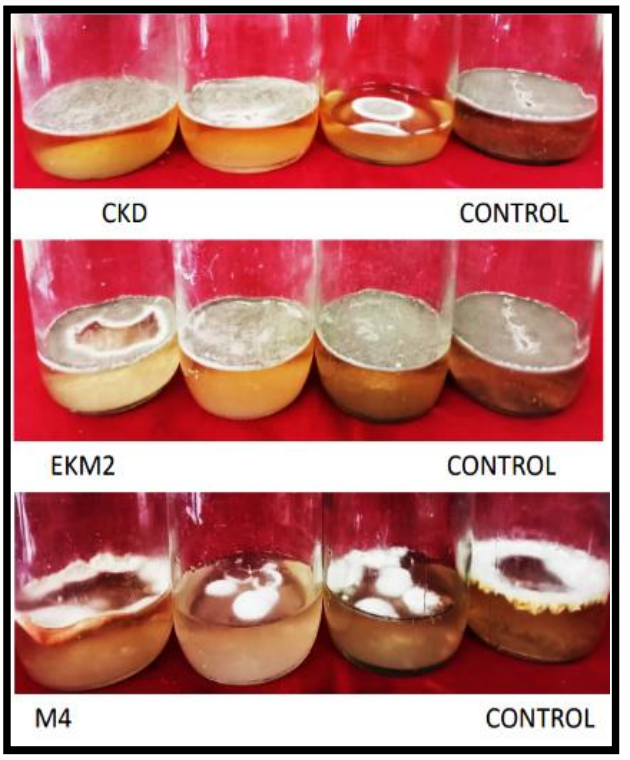
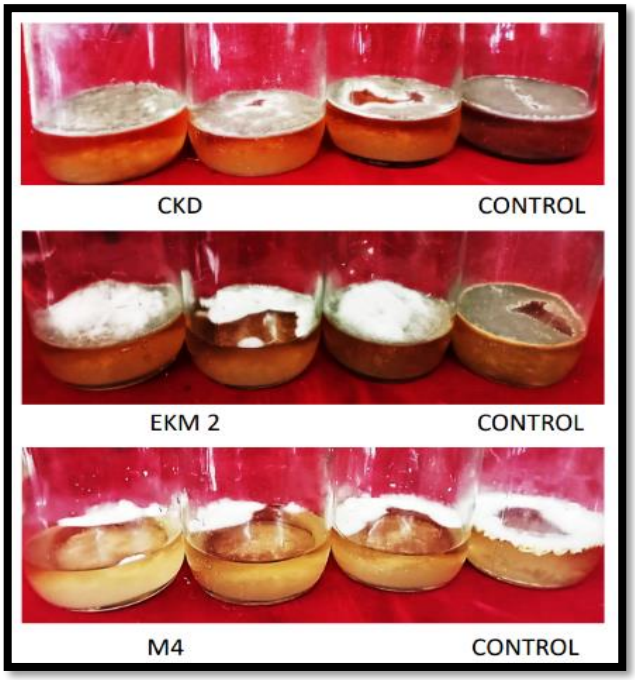


25%

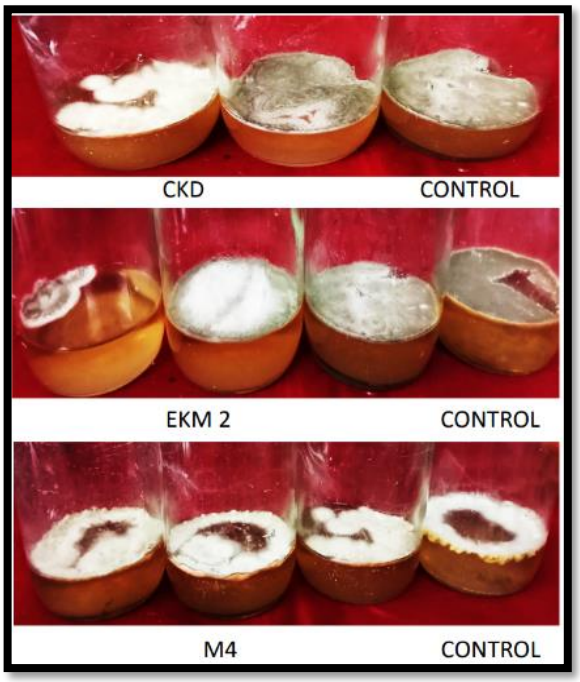


27.5%

Plate 9. Growth and sporulation of *Metarhizium anisopliae* isolates at different concentrations of Polyethylene ethylene glycol (PEG)



30%



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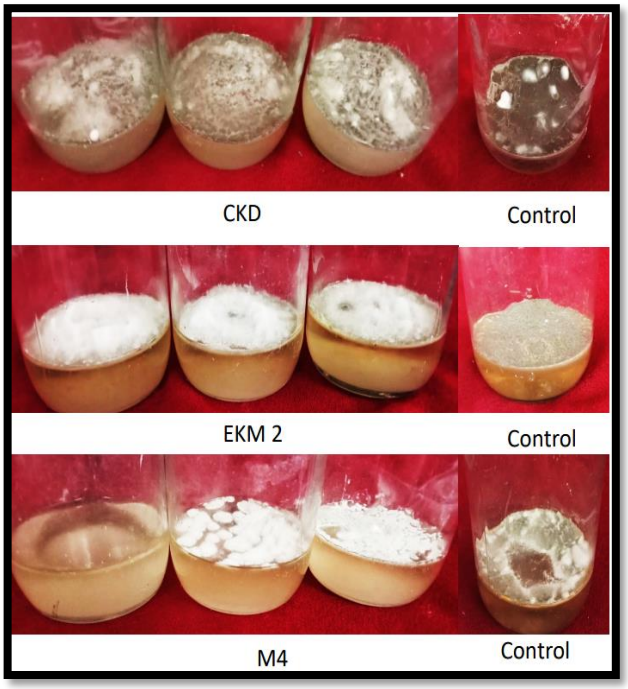

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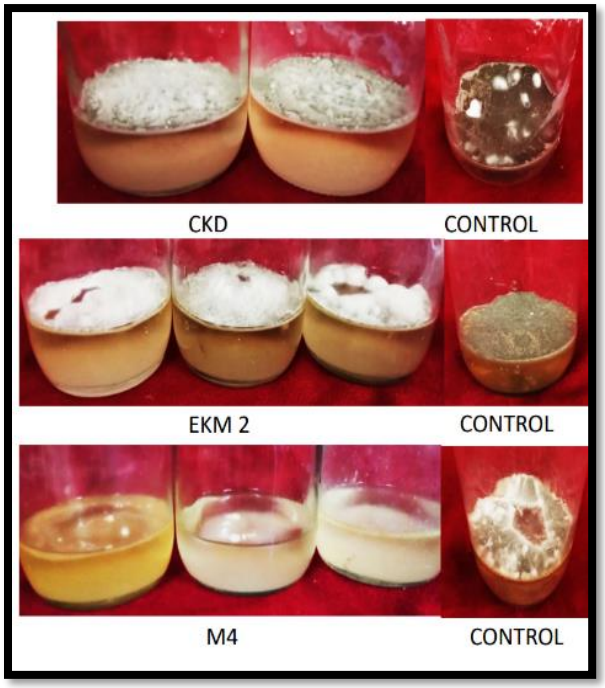

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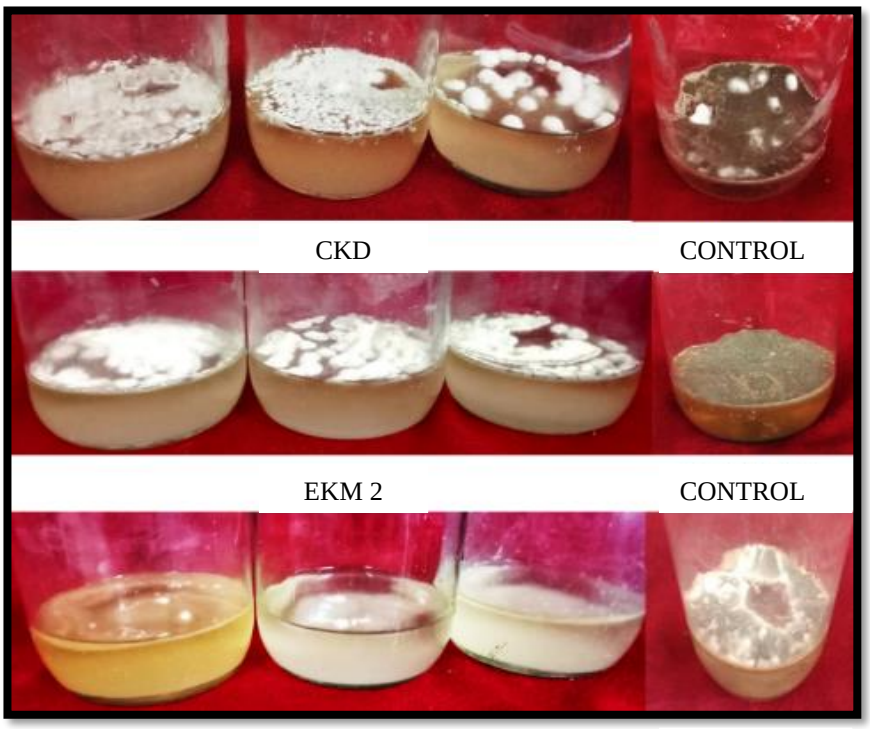
Plate 10. Growth and sporulation of *Metarhizium anisopliae* isolates at different concentrations of Polyethylene ethylene glycol (PEG)



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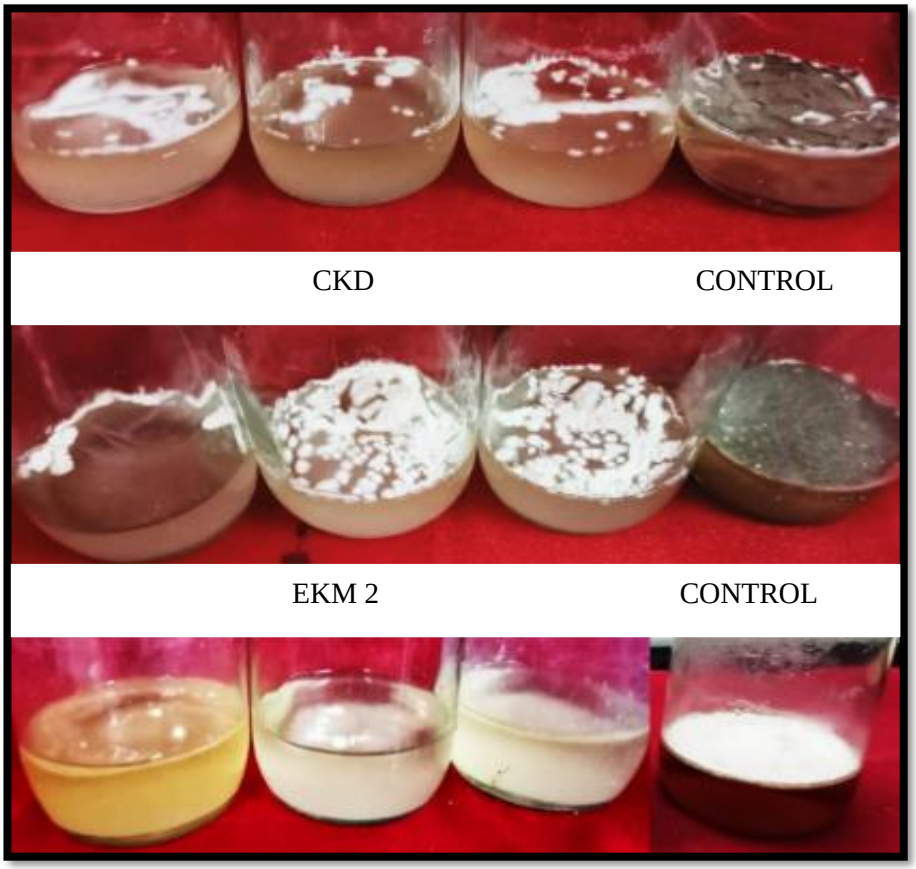
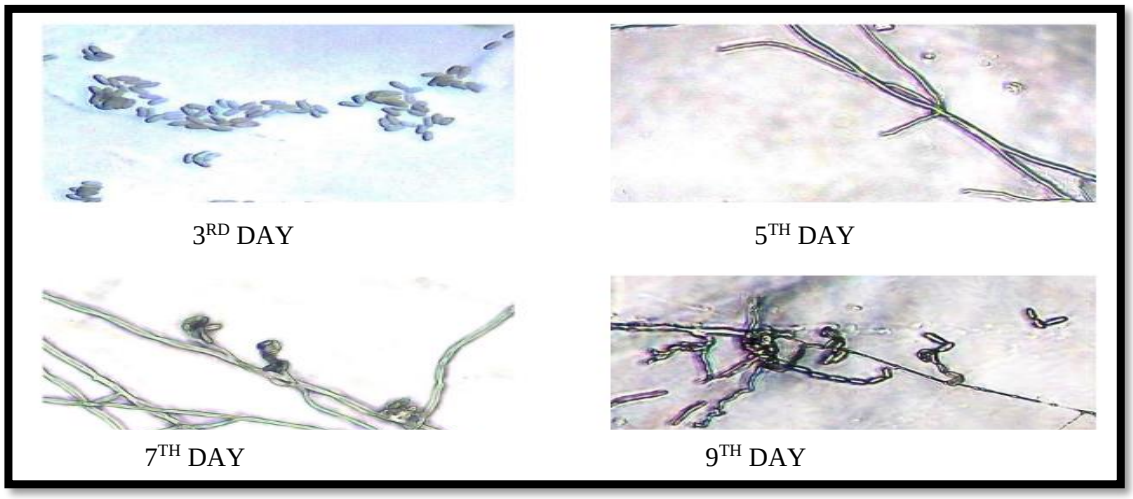
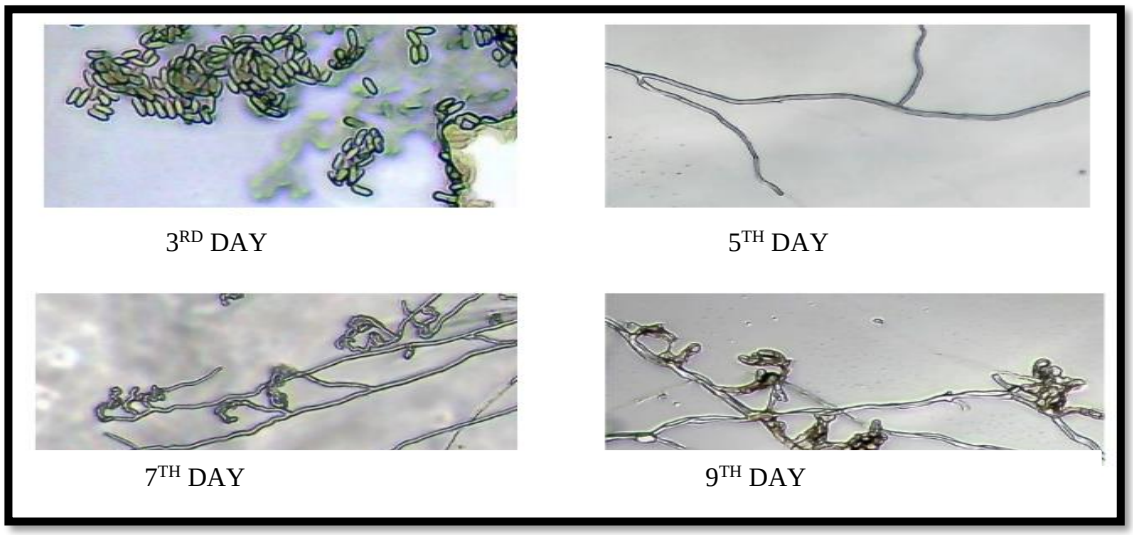


Plate 11. Growth and sporulation of *Metarhizium anisopliae* isolates at different concentrations of Polyethylene ethylene glycol (PEG)





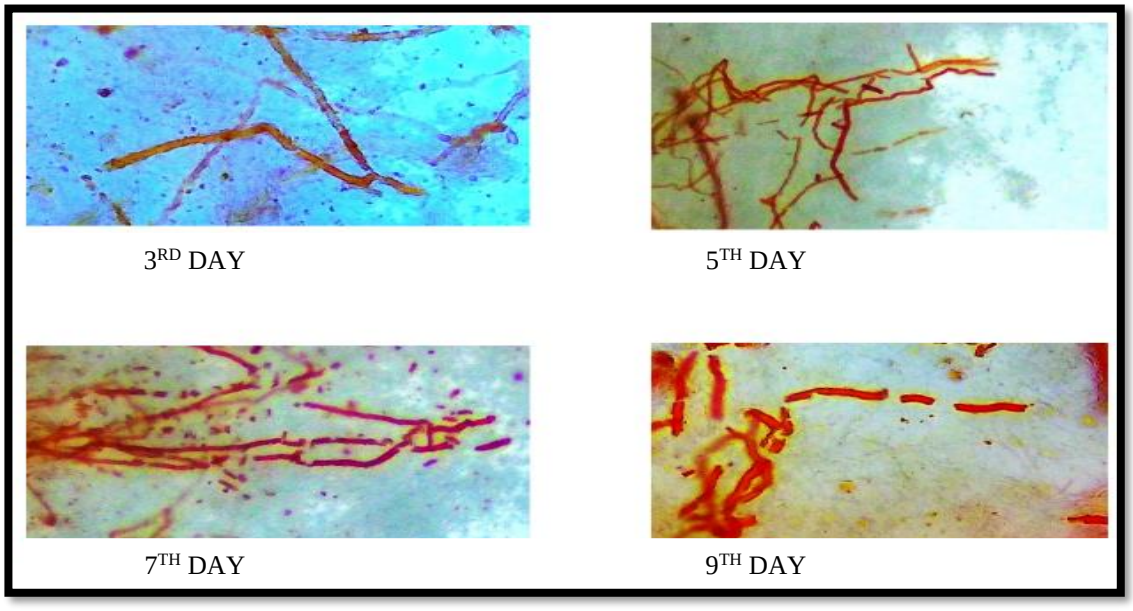


Plate 12. Effect of 39 % PEG on spore size and mycelial thickness of *Metarhizium anisopliae* isolates

EKM2 (7.48g) followed by CKD (6.38g), which was higher compared to previous treatments. The isolate, M4 has shown a mycelial weight of 2.36g which was significantly lower when compared to the other two isolates (Plate 15). The same trend was followed at the temperature levels of 38ºC and 39ºC (Plate 16 and 17). From 40ºC onwards the mycelia weight of all the isolates were reduced compared to previous treatments. At 40ºC, EKM 2 has recorded the highest mycelial weight (2.13g) followed by CKD (1.48g). The lowest weight of mycelia (0.63g) was observed in the isolate M4 (Plate 19). At the highest temperature level of 41ºC, EKM 2 has shown a mycelial weight of 1.33g which was highest among the three isolates. It was followed by CKD which has shown a weight of 1.151g and the lowest was recorded in the isolate M4, (0.63g). Highest temperature level tolerated by all the three isolates was 41ºC and none of the isolates had shown growth at temperature above 41ºC (Plate 20).

Through temperature inducing method, all the isolates showed improved growth and more stress tolerance. From the experiment it is clear that EKM2 has recorded highest weight of mycelia at all the temperature levels ranging from 1.36 g to 12.75g with moderate amount of sporulation and it was followed by CKD with mycelial weight ranging from 1.15g to 6.38g, but the sporulation was more compared to EKM2. In all the treatments, M4 has recorded the lowest mycelial weight (0.63g – 4.46g) with least amount of sporulation (Plate 13 and 21). Hence it is proved that EKM2 is superior and tolerant to thermal stress and the isolate M4 has been identified as the susceptible isolate.

Table 10. Effect of induced temperature on mycelial weight of *Metarhizium anisopliae* isolates

Mean mycelia weight at different temperature level (g/100ml)									
Isolates	**Control (28±2ºC)**	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**	**Mean**
CKD	4.914^{f}	4.837^{fg}	3.428^{i}	6.382^{d}	5.659^{e}	4.642^{fg}	1.486^{j}	1.151^{klm}	4.062^{b}
EKM2	14.327^{a}	12.475^{b}	12.750^{b}	7.488^{c}	6.694^{d}	6.293^{de}	2.139^{i}	1.336^{kl}	7.934^{a}
M4	3.612^{hi}	4.214^{gh}	3.675h^{i}	2.360^{j}	4.463^{j}	2.161^{j}	0.638^{lm}	0.638^{m}	2.495^{c}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

4.7.2.2. Effect of induced temperature on per centage inhibition of *Metarhizium anisopliae* isolates

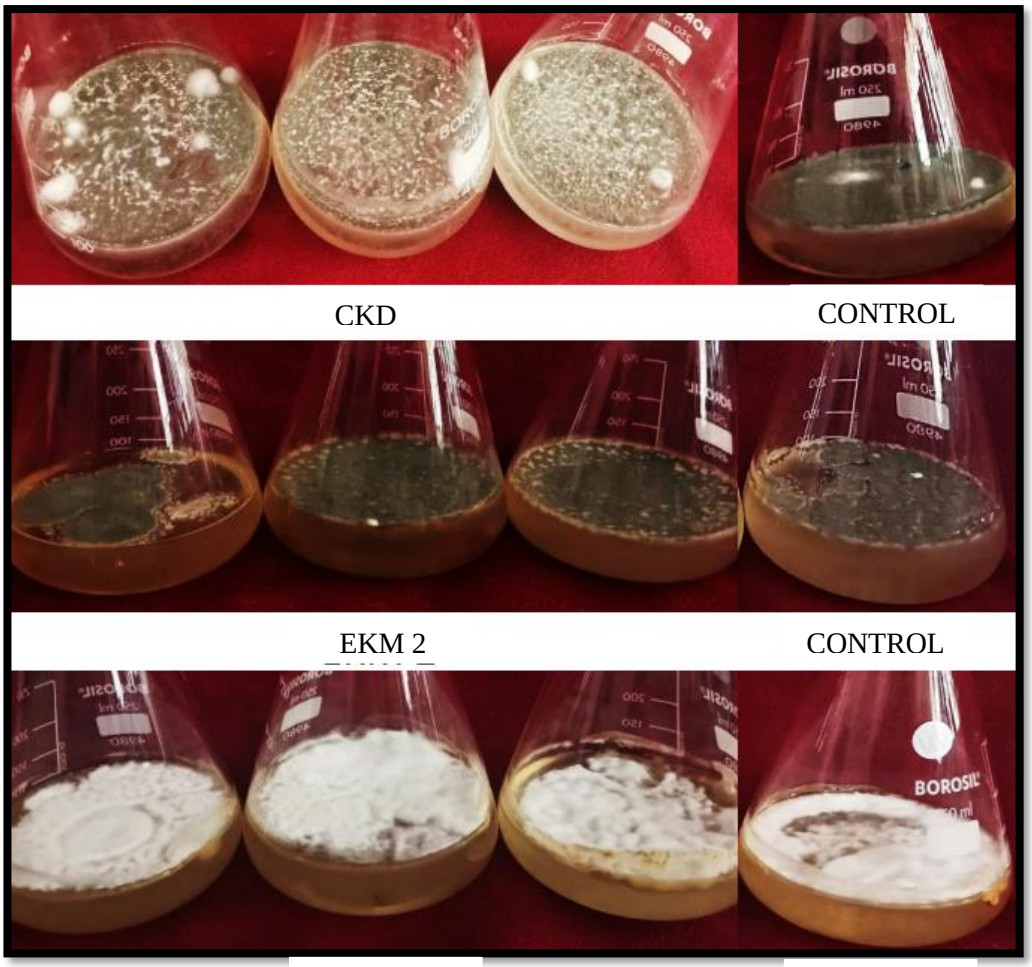
The radial growth of the colony after ten days of incubation in the PDA plates were recorded and the per cent growth inhibition at all the temperature levels were calculated. The results of the experiment are depicted in the Table 11.

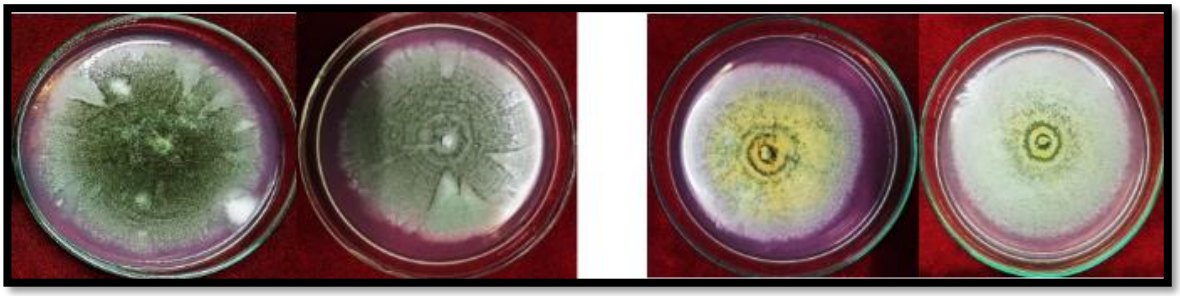
Table 11. Effect of temperature on inhibition of *Metarhizium anisopliae* isolates

Mean percentage inhibition at different temperature level (%)							
Isolates	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**
CKD	18.517^{j}	31.477^{i}	55.550^{gh}	56.290^{gh}	60.740^{efg}	55.553^{gh}	64.810^{def}
EKM2	19.440^{j}	58.330^{fgh}	51.856^{h}	71.107^{cd}	77.770^{bc}	92.587^{a}	0.000^{l}
M4	11.110^{k}	12.960^{jk}	33.330^{i}	67.590^{de}	79.997^{b}	64.810^{def}	0.000^{l}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

The lowest growth inhibition at 35ºC was shown by M4 (11.11%) followed by CKD (18.5%). The highest percent inhibition was recorded in the isolate EKM2 (Plate 9). At 36ºC, lowest inhibition and more growth was observed in the isolate M4 (33.33%) followed by CKD (51.35%) and the highest growth inhibition was recorded in the isolate EKM2 (55.55%) (Plate 11). At 37ºC also, same trend was observed. At 38ºC, the highest inhibition was shown by the isolate EKM 2 (71.10%). The lowest inhibition was shown by the isolate CKD (56.29%) followed by M4 (67.59%) (Plate 12). When the temperature was increased to 39ºC, the highest growth inhibition was shown by the isolate M4 (79.99%) (Plate 13) and the lowest inhibition was observed in the isolate CKD (60.74%) followed by EKM2 (77.77%) (Plate 14). At an enhanced temperature of 40ºC, the highest growth inhibition was shown by EKM2 and was followed by CKD and the lowest inhibition was recorded by M4 (Plate 15). At the highest temperature of 41ºC, the isolate CKD has shown some growth with an inhibition of 64.81% while isolates CKD and M4 did not record any growth (Plate 16). At 42ºC, none of the isolate has shown growth. The sporulation of CKD was higher at all levels of temperature for the isolate CKD and the isolate EKM2 has





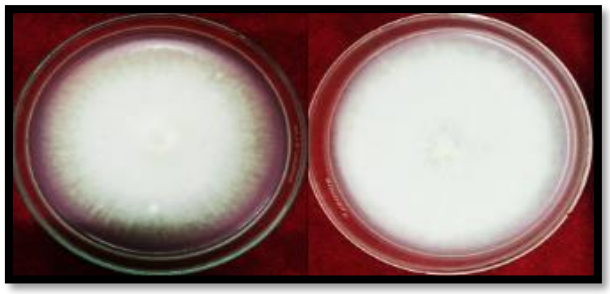


Plate 13. Effect of induced temperature (35 oC) on growth and sporulation of *Metarhizium anisopliae* isolates

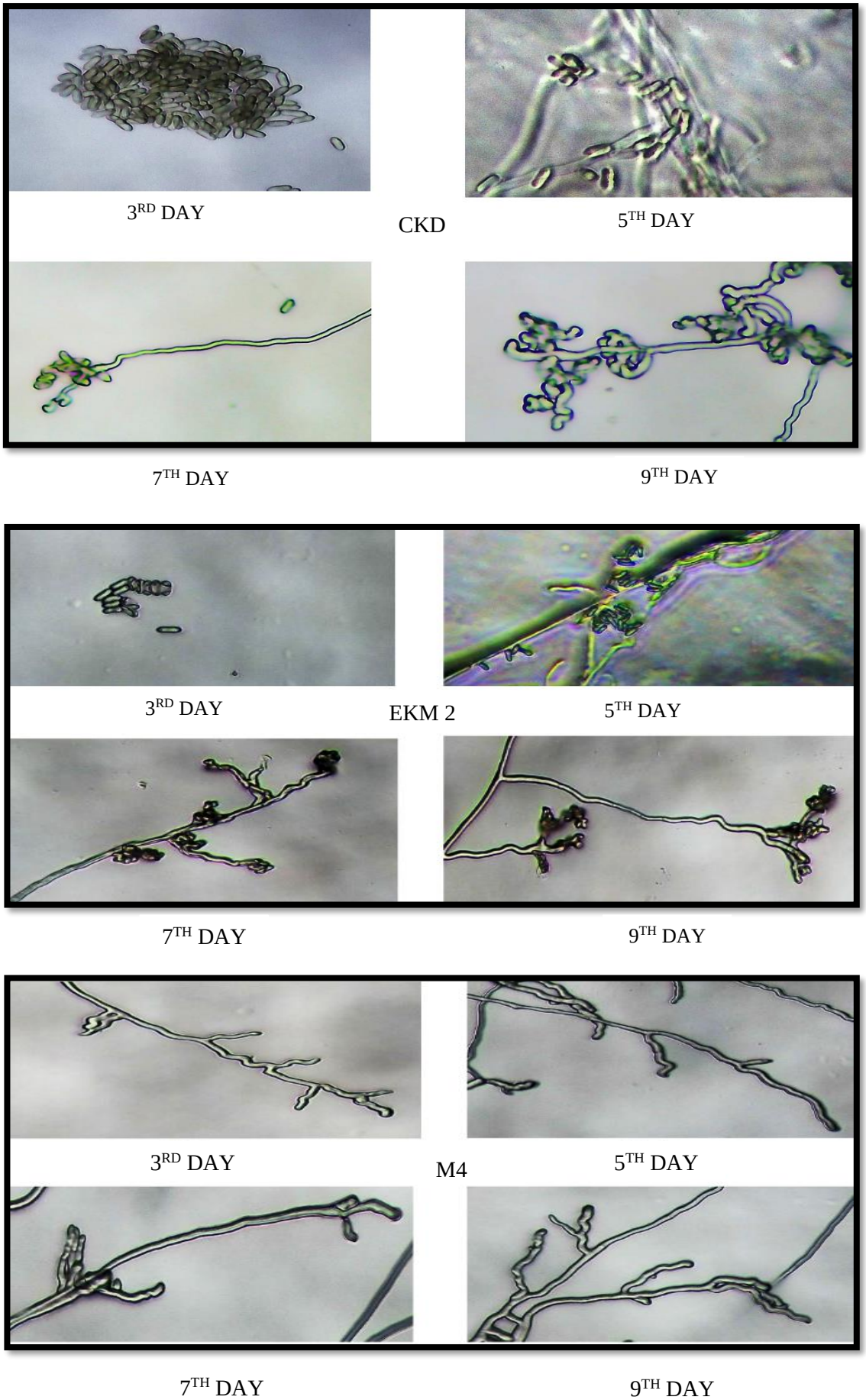


Plate 14. Effect of induced temperature (35°C) on spore size and mycelial thickness of *Metarhizium anisopliae* isolates

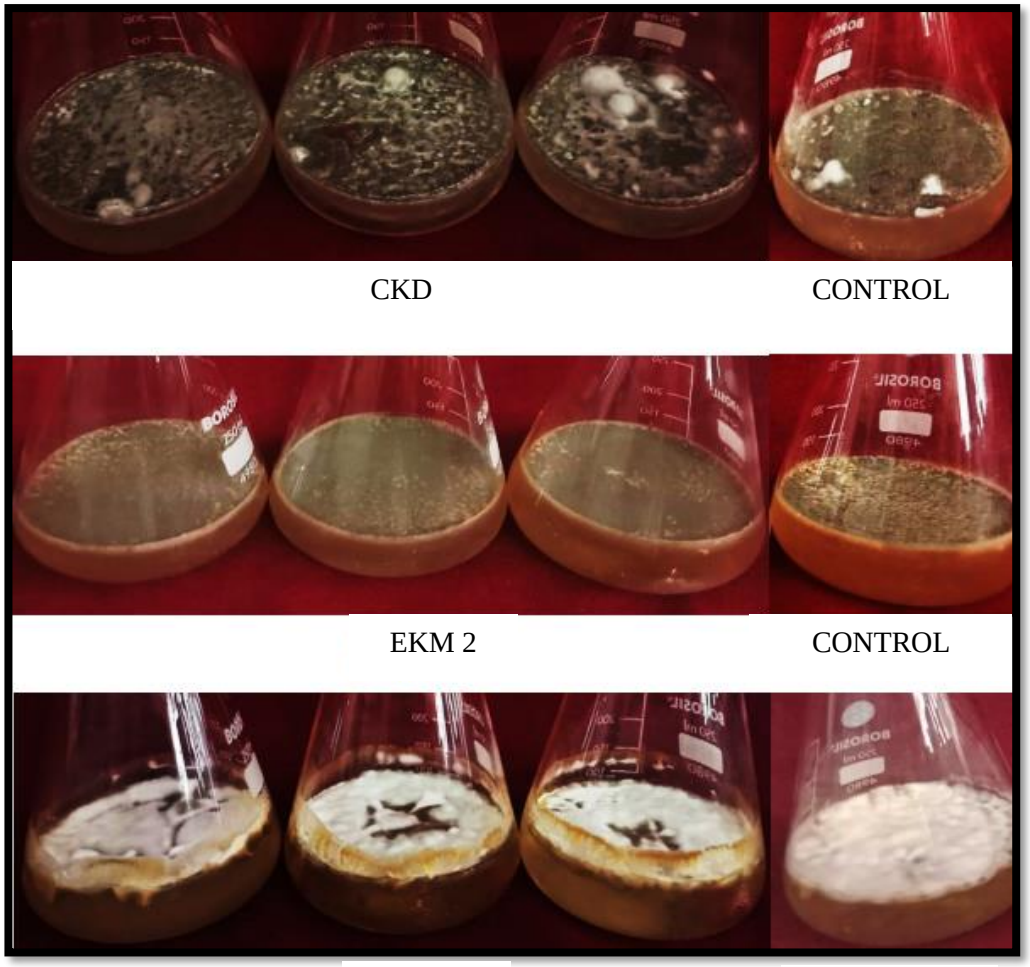
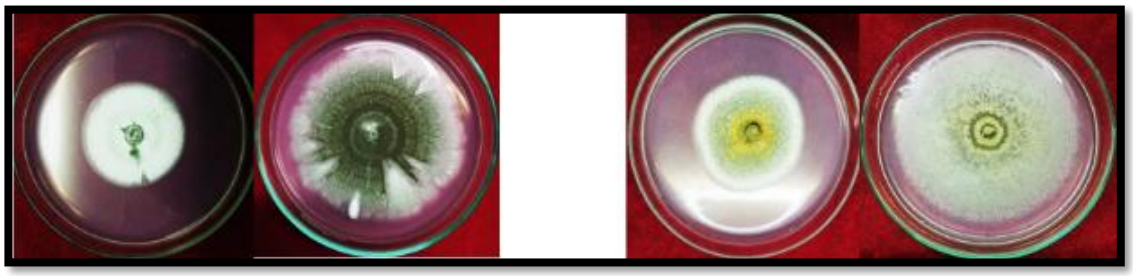


Plate 15. Effect of induced temperature (36°C) on growth and sporulation of *Metarhizium anisopliae* isolates



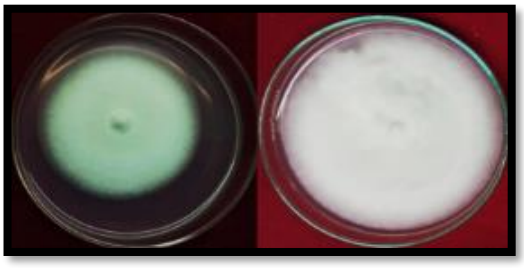
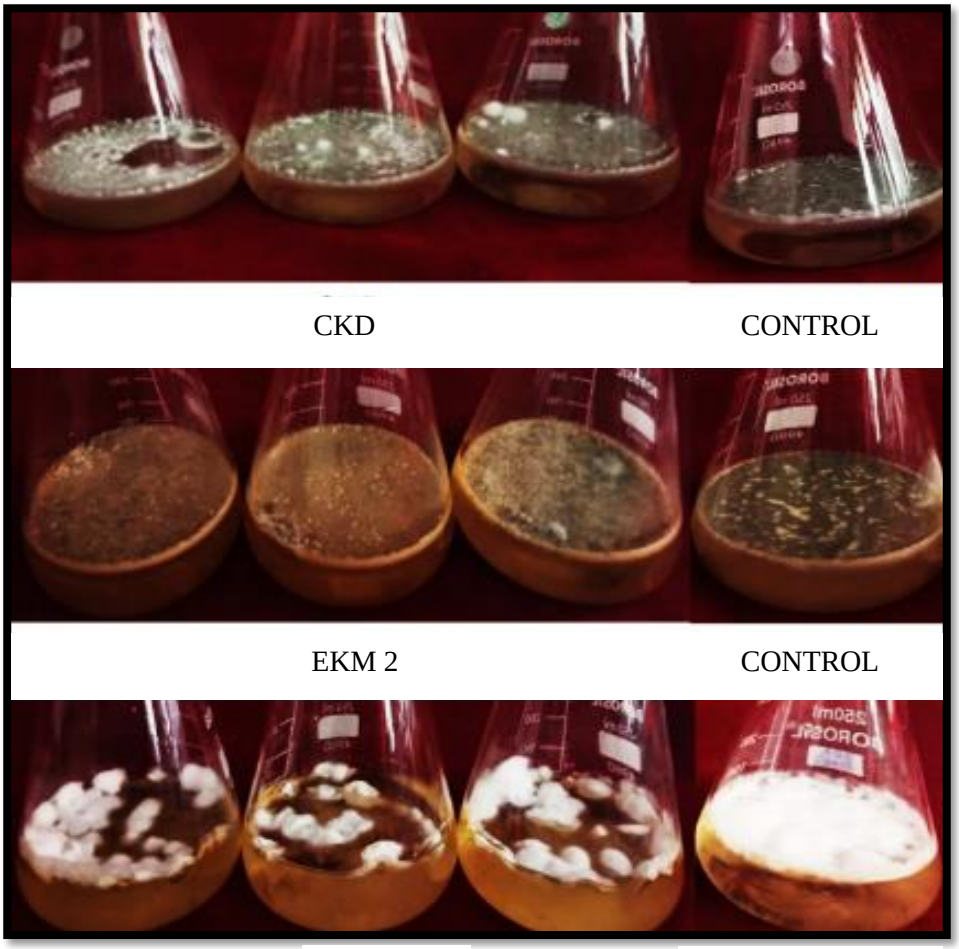


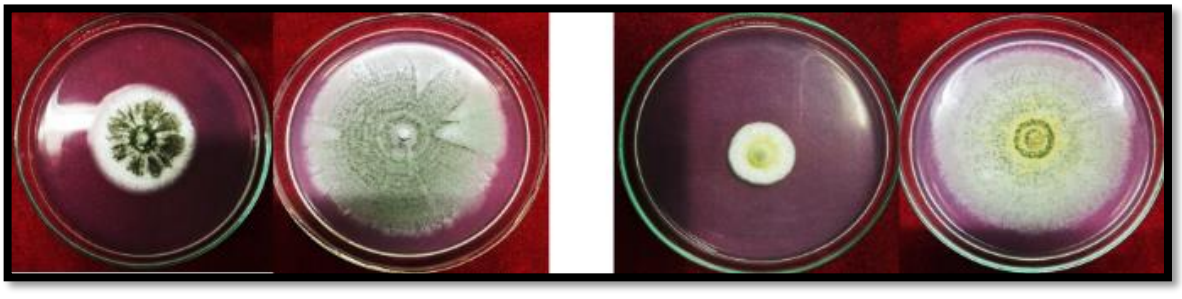
Plate 16. Effect of induced temperature (37ºC) on growth and sporulation of *Metarhizium anisopliae* isolates



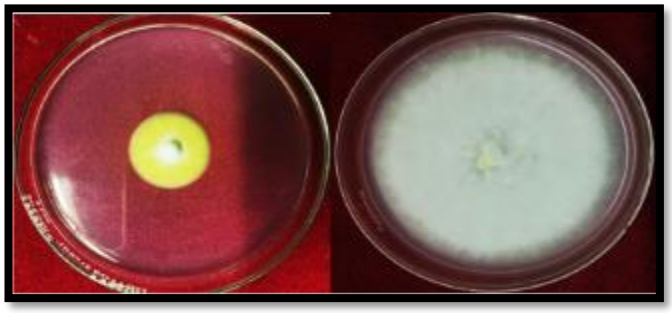
CKD CONTROL

EKM 2 CONTROL

M4 CONTROL



CKD EKM 2



M4

Plate 17. Effect of induced temperature (38^{o}C) on growth and sporulation of *Metarhizium anisopliae* isolates

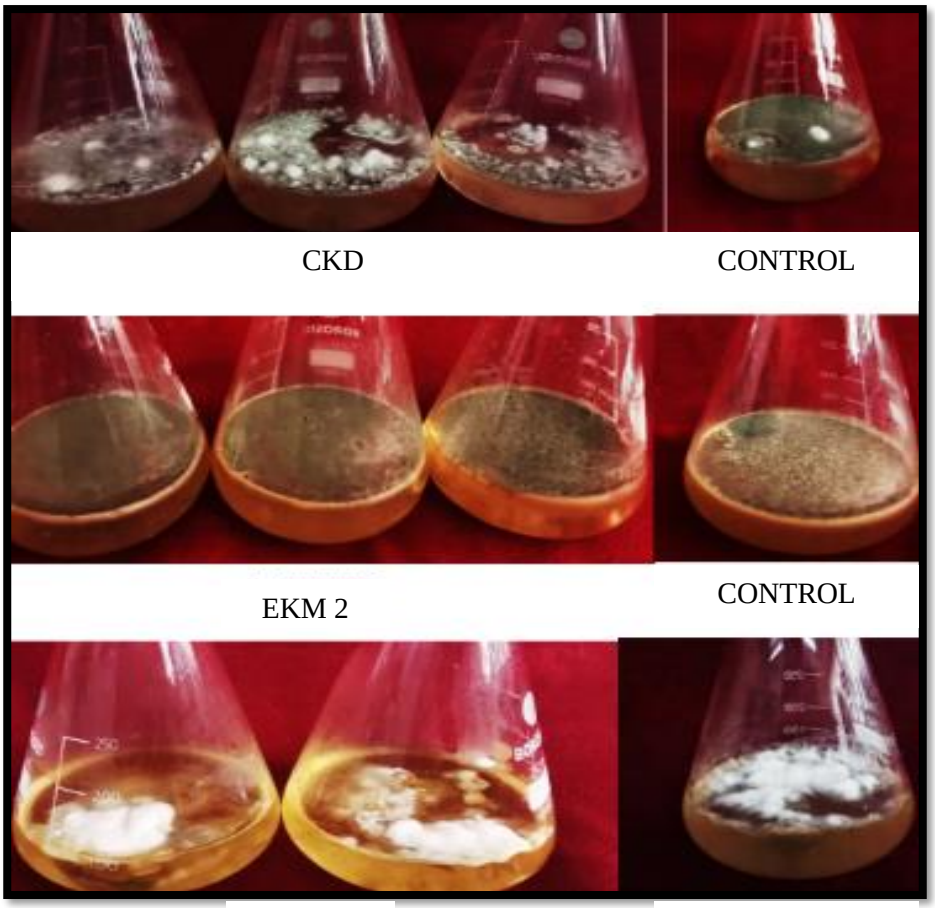
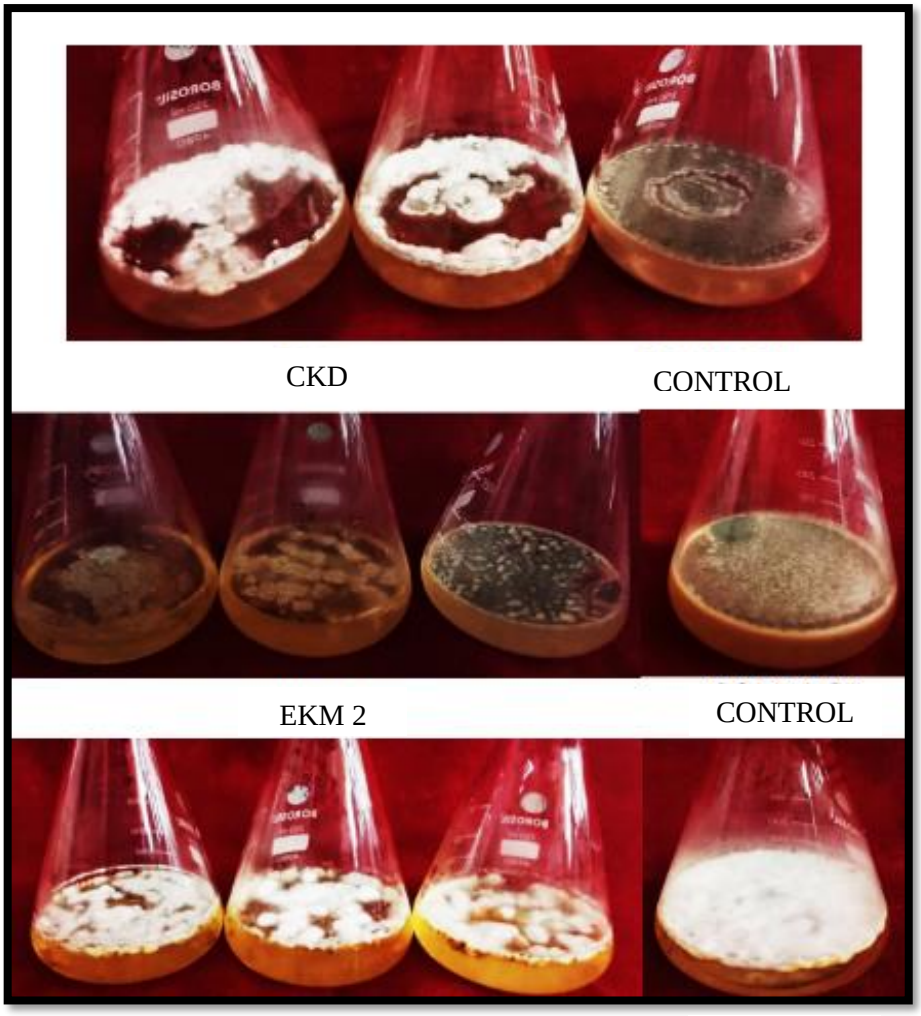


Plate 18. Effect of induced temperature (39^{o}C) on growth and sporulation of *Metarhizium anisopliae* isolates



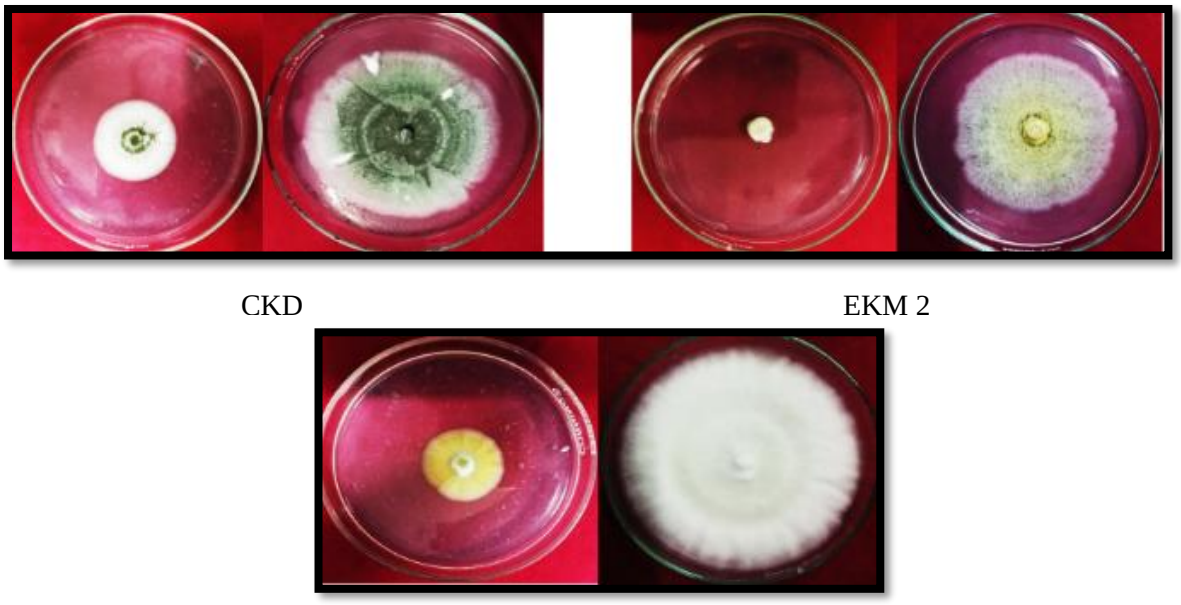
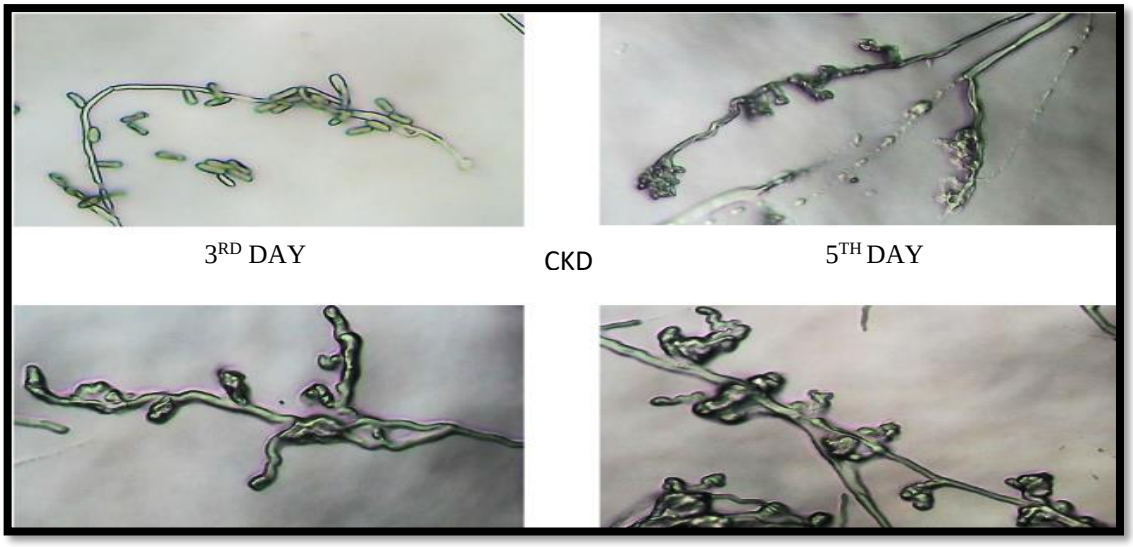
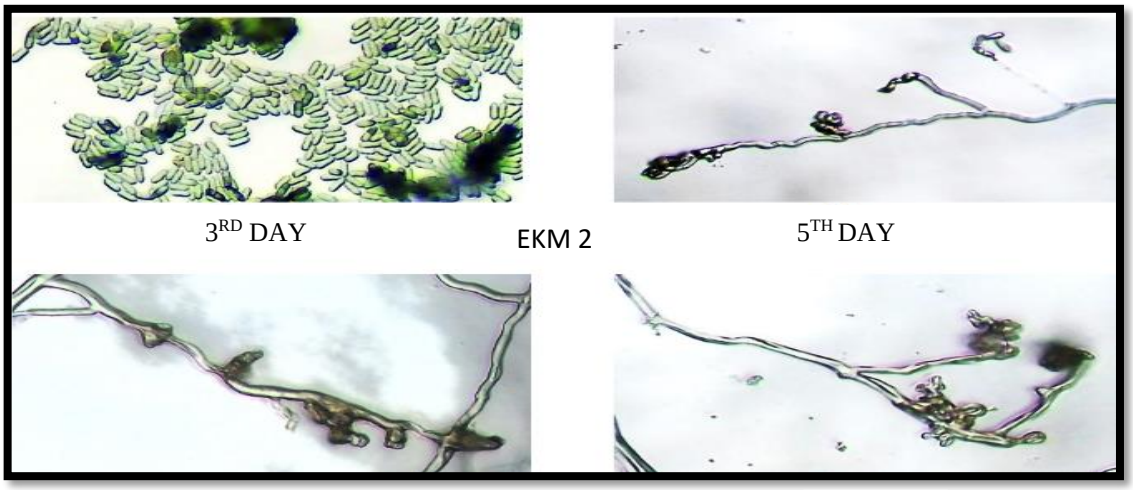


Plate 19. Effect of induced temperature (40ºC) on growth and sporulation of *Metarhizium anisopliae* isolates

Plate 20. Effect of induced temperature (41^{o}C) on growth and sporulation of *Metarhizium anisopliae* isolates





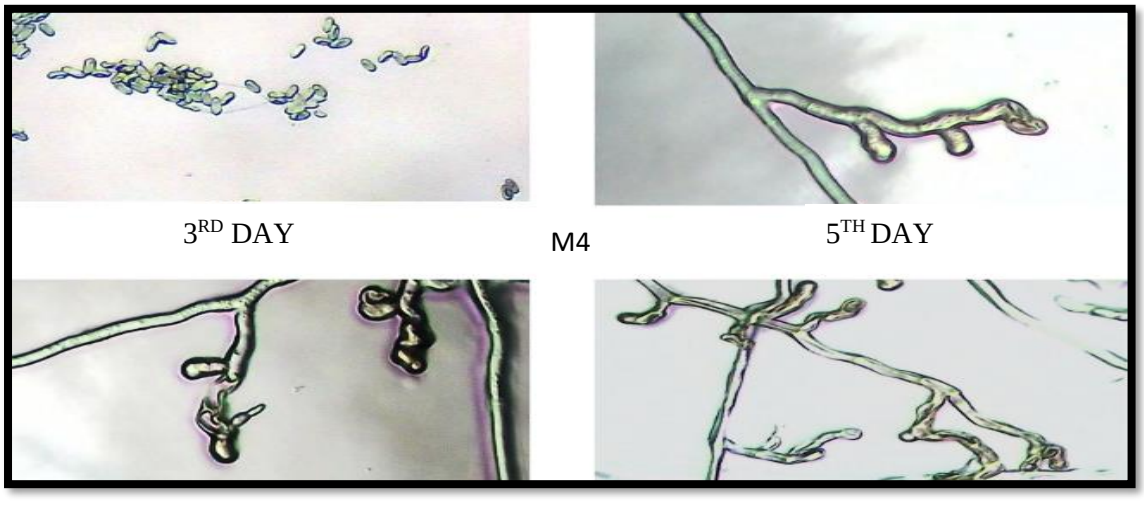
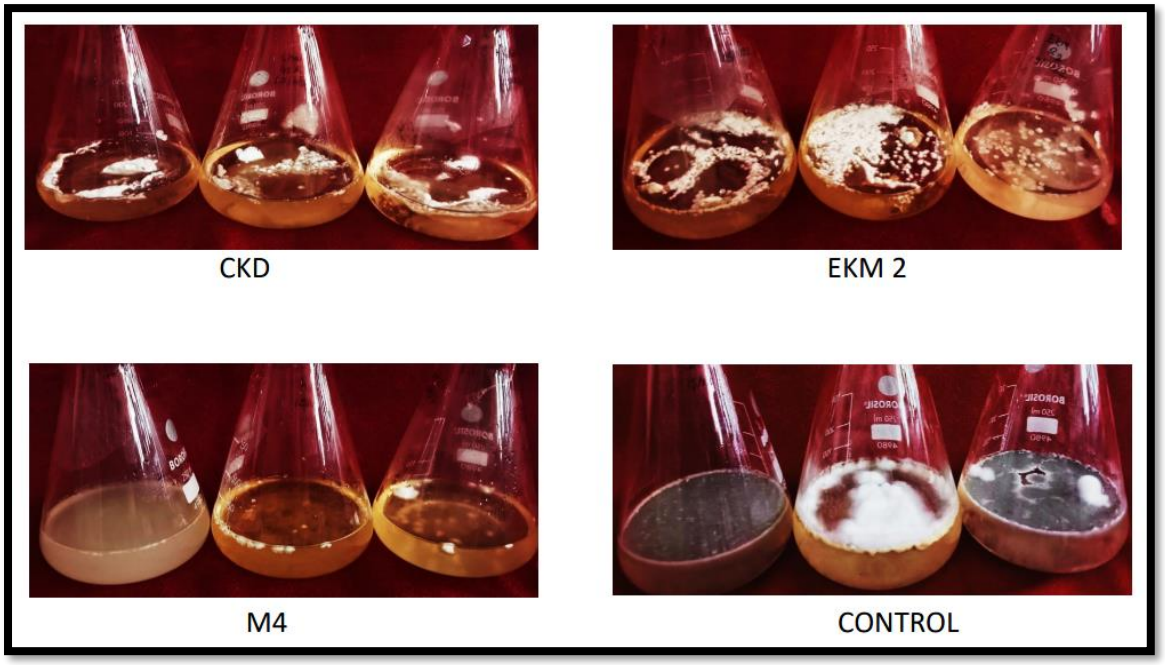
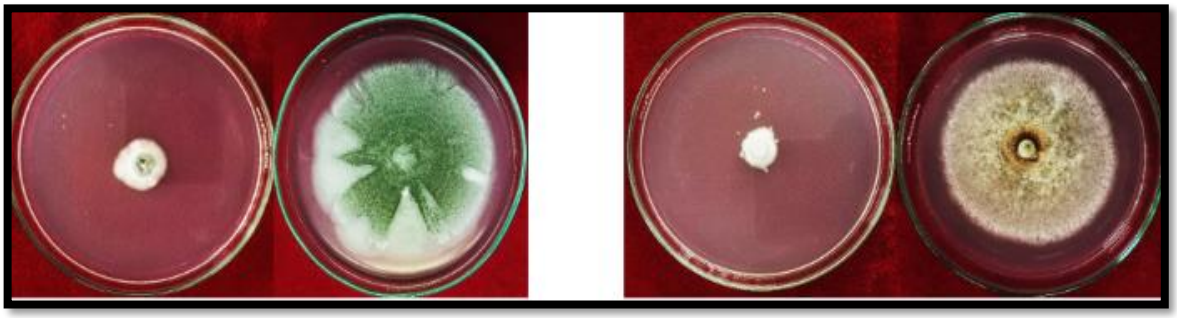


Plate 21. Effect of induced temperature (41°C) on spore size and mycelial thickness of *Metarhizium anisopliae* isolates





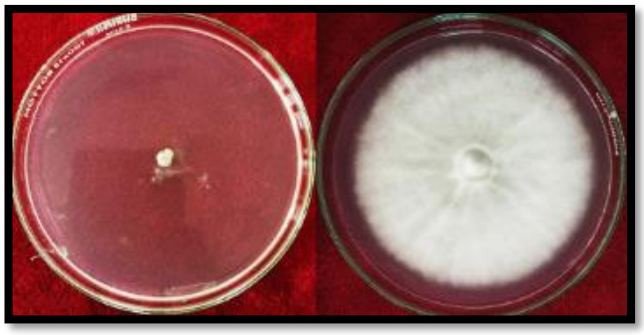


Plate 22. Effect of induced temperature (42ºC) on growth and sporulation of *Metarhizium anisopliae* isolates

shown a moderate level of sporulation. The isolate M4 has shown least amount of sporulation at all the treatments (Plate 18).

4.7.2. Effect of induced temperature on spore germination of *Metarhizium anisopliae* isolates

The spore germination at different levels of temperature was found by calculating the colony forming units (CFU)/ ml. The number of colonies at the 3rd dilution was counted and the results are depicted in the Table 12.

Table 12. Effect of induced temperature on spore germination of *Metarhizium anisopliae* isolates

Mean spore germination at different temperature levels (cfu/ml)*								
Isolates	**Control (28±2ºC)**	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**
CKD	257.000^{b}	201.667^{e}	194.667^{e}	182.00^{fg}	179.667^{g}	141.333^{i}	32.667^{lm}	11.000^{op}
EKM2	280.333^{a}	230.667^{c}	218.667^{d}	215.000^{d}	192.000^{ef}	169.333^{h}	49.667^{k}	18.667^{no}
M4	62.667^{j}	40.000^{kl}	38.000^{lm}	35.667^{lm}	30.667^{lm}	28.667^{mn}	15.333^{o}	3.000^{p}

* Number of colonies X 10^3/ml (CFU/ ml at 3rd dilution)*****Mean of three observations

At 35ºC, the highest spore germination was observed in the isolate EKM2 (230.667 CFU /ml) followed by CKD (201.667 CFU/ml). The lowest number of colonies was observed in the M4 (40 CFU/ml). When the temperature was enhanced to 36ºC, EKM 2 recorded the highest number of colonies (218.667 CFU/ml), followed by CKD (194.667 CFU/ml). M4 has shown the lowest number of colonies (38 CFU/ml) at 36ºC. The isolate, EKM 2 had recorded the highest number of colonies (215 CFU/ml) followed by CKD (182 CFU/ml) at an increased temperature of 37^0C. The isolate M4 recorded the lowest number of colonies (35.667 CFU/ml) at 37ºC. Same trend was followed at 38ºC with the highest number of colonies recorded by the isolate EKM2 (192 CFU/ml) followed by CKD (179.667 CFU/ml) and the least number of colonies was recorded by the isolate M4 (30.667 CFU/ml). At a higher temperature of 39ºC, highest number of colonies (169 CFU/ml) was observed in the isolate EKM2 followed by CKD (141 CFU/ml). The lowest number of colonies was

observed in isolate M4 with 28 CFU/ml. From 40ºC onwards the number of colonies produced / ml was reduced drastically. The isolate EKM2 has recorded a higher number of colonies (49 CFU/ml) followed by CKD (39 CFU/ml). The isolate, M4 recorded the lowest number of colonies at 40ºC (15 CFU/ml). At 41ºC, all the isolates produced fewer colonies. The isolate, EKM2 produced only 18 CFU/ml followed by CKD (11 CFU/ml) and M4 (3 CFU/ml).

The results showed that, at all the levels of temperature, EKM2 has recorded the highest number of colonies/ ml followed by CKD. The isolate M4 was the one with the lowest number of colonies/ ml. The spore germination, was found to be the highest in the isolate EKM2 at all temperature levels, hence proved as thermo tolerant isolate. The isolate CKD was also showing higher spore germination but less compared to EKM2. The isolate EKM2 was moderately tolerant to temperature. The isolate M4 with the lowest spore germination at all temperatures has been identified as the susceptible one.

4.7.3. Effect of induced temperature on spore count of *Metarhizium anisopliae* isolates

The spore count at all the temperature levels was counted from the broth using Hemocytometer. The results of the experiment are shown in table 13.The isolate EKM2 recorded the highest spore count at all temperature levels. At 35ºC, highest spore count (5.4 X 10^{7}/ml) was recorded by EKM2 followed by CKD (4.2 X 10^{7}/ ml). The lowest spore count was found in isolate M4 with 0.633 X 10^{7} spores/ml.

Table 13. Effect of induced temperature on spore count of *Metarhizium anisopliae* isolates

Mean spore count at different temperature level (spores/ml)*								
Isolates	**Control (28±2ºC)**	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**
CKD	19.497^{a}	4.247^{e}	7.400^{d}	1.433^{fg}	0.967^{fgh}	0.683^{gh}	0.550^{gh}	0.483^{gh}
EKM2	14.200^{b}	5.400^{e}	9.167^{c}	1.950^{f}	1.350^{fgh}	1.000^{fgh}	0.733^{gh}	0.783^{fgh}
M4	4.283^{e}	0.633^{gh}	0.567^{gh}	0.633^{gh}	0.450^{gh}	0.200^{h}	0.217^{h}	0.217^{h}

*Spore count X 10^{7} /ml**Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

At 36ºC, also highest spore count was observed in the isolate EKM2 (9.16 X 10 ^{7}/ml) followed by CKD (7.4 X 10 ^{7}/ml), while M4 recorded the lowest spore count (0.56 X 10 ^{7}/ml). When the temperature was increased to 37ºC, EKM2 showed the highest number of spores (1.9 X 10 ^{7}/ml) followed by CKD (1.43 X 10 ^{7}/ml). The isolate M4 has shown the lowest number of spores (0.63 X 10 ^{7}/ml). At an enhanced temperature of 38ºC, EKM2 showed a spore count of 1.35 X 10 ^{7}/ml followed by CKD which were on par with each other (0.967 X 10 ^{7}/ml). The lowest was shown by isolate M4. Higher temperature of 39ºC resulted in the highest spore count by EKM 2 (1 X 10 ^{7}/ml) followed by CKD (0.97 X 10^{7}/ml). The lowest spore count was observed in isolate M4 (0.2 X 10 ^{7}/ml). When the temperature was elevated to 40ºC, the highest spore count of 0.733 X 10 ^{7}/ml was observed in the isolate, EKM2 followed by CKD with 0.55 X 10 ^{7}/ml. The lowest spore count was recorded by the isolate M4 (0.217X 10^{7}/ml). At the highest temperature of 41ºC, number spores were found highest in the isolate EKM2 (0.783 X 10 ^{7}/ml) followed by CKD (0.483 X 10 ^{7}/ml). The least number of spores was recorded by the isolate M4 (0.217 X 10 ^{7}/ml).

The results showed that at all temperature levels, EKM2 has recorded the highest number of spores/ ml of PD broth. CKD has also shown a higher number of spores/ ml at all temperatures. The lowest number of spores at all temperature levels was observed in the isolate M4. So EKM2 has been identified as the thermo-tolerant isolate and M4 as the susceptible one. The isolate CKD was also moderately tolerant to temperature.

4.7.4. Effect of induced temperature on size and shape of the spores of *Metarhizium anisopliae* isolates

The spore size at all levels of temperatures were measured by observing under an image analysing compound microscope. The size was measured in micrometers using the computer software Radical (Table. 14). The shape of the spores was also observed using a compound microscope.

At the lowest temperature of 35ºC, highest length of spores was observed in the isolate EKM2 (8.03 µm) followed by CKD (7.35 µm) (Plate 13). The lowest length of spores was observed in isolate M4 (6.08 µm). At 36ºC also, higher spore length was recorded in the isolate EKM2 (7.15 µm) followed by CKD (3.90 µm). The isolate M4 was with lowest spore length (3.24 µm).

At 37ºC, EKM2 has shown the highest spore length of 7.02 µm followed by CKD with a spore length of 3.19 µm. At 38ºC, the isolate CKD has shown the highest spore length (6.74 µm) followed by EKM2 (5.22 µm). Among the three *Metarhizium* isolates, M4 was the one that showed a smaller spore length (2.69 µm). Higher temperature of 39ºC resulted in the highest spore size of 5.213 µm by the isolate, EKM2 and it was followed by CKD (4.313µm) and the least length of spores was observed in isolate M4 (2.77µm). The same trend was followed at 40ºC and 41ºC. When the temperature was elevated to 40ºC, EKM2 has shown a spore length of 3.47 µm followed by CKD (2.81 µm), and the smallest size of spores was observed in the isolate M4 (1.98 µm). At the highest temperature of 41ºC, EKM 2 has recorded a higher length of spores (3.47 µm) followed by CKD (3.18 µm). The least size of spores was observed in the isolate M4 (1.63 µm) (Plate 21).

The shape of the spores at different temperatures was also recorded. The observations are depicted in the Table 15.

At 35ºC, the isolate CKD showed oblong, oval, and dumbbell-shaped spores. The shape of EKM2 spores was elongated, oval-shaped with segmentation at the two edges. The isolate M4 produced longer, elongated, oval, and thick-walled spores (Plate 12). At 36ºC, CKD was with oval-shaped and long thick-walled spores. The isolate EKM2 was oval to round, broad-shaped with thicker walls. The isolate M4 has shown spores that were large with dumbbell shapes. Elongated and oblong-shaped spores were recorded for CKD at 37ºC, while EKM2 had spores that are long and little shrunken and the isolate M4 showed long thick-walled spores.

Table 14. Effect of induced temperature on size of the spores of *Metarhizium anisopliae* isolates

Mean spore size at different temperature levels (µm)								
Isolate	**Control**	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**
CKD	13.017^{a}	7.350^{bc}	3.960^{fg}	3.197^{hi}	6.747^{cd}	4.313^{f}	2.810^{hij}	3.180^{hi}
EKM2	13.013^{a}	8.033^{b}	7.150^{c}	7.023^{c}	5.277^{e}	5.213^{e}	4.530^{ef}	3.470^{gh}
M4	6.970^{c}	6.087^{d}	3.247^{ghi}	2.733^{hijk}	2.690^{ijk}	2.77^{jkl}	1.987^{kl}	1.637^{l}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

At 38ºC, CKD was with long dumbbell-shaped spores and EKM2 was with long thick-walled spores with little shrinkage. When the temperature was raised to 39ºC, CKD was with small shrunken spores with oblong shapes while EKM2 has shown small shrunken spores with thick walls. At an elevated temperature of 40ºC, the size of the spores was smaller compared to previous treatments. Both CKD and EKM 2 have small-sized abrupt shaped spores with thicker walls. At the maximum temperature of 41ºC, the isolates recorded very small-sized shrunken spores with thicker walls that are clustered together.

At all temperature levels, EKM2 has recorded the highest spore length followed by CKD. A smaller spore length was observed at all temperatures in the isolate M4. Hence EKM2 has been identified as thermo tolerant and M4 has proved as susceptible to temperature stress. The isolate CKD was moderately tolerant to temperature (Table 15)

4.7.5. Effect of induced temperature on mycelial thickness of *Metarhizium anisopliae* isolates

At 35ºC, the highest thickness of mycelia was observed in the isolate EKM2 (7.16 µm) followed by CKD (5.24 µm). The least thickness of mycelia was observed in isolate M4 (3.68 µm) (Plate 13). At 36ºC, highest thickness was observed in the isolate EKM 2 (6.49 µm) followed by CKD (3.58 µm). The mycelial thickness of M4 at 36ºC was 3.533 µm. At 37ºC, EKM2 has shown a mycelial thickness of 4.42 µm followed by CKD (3.51 µm) and M4 has shown the lowest thickness of 2.32 µm. At a higher temperature of 38ºC, highest mycelial thickness was observed in the isolate EKM2 (4.33 µm) followed by CKD (3.66 µm). The isolate M4 has recorded the lowest mycelial thickness of 2.75 µm at 38ºC. Maximum mycelial thickness of 3.87 µm was recorded by the isolate EKM2 at 39ºC and was followed by the isolate CKD with 3.45 µm thickness. The isolate M4 has shown a thickness of 2.69 µm at the same temperature. When the temperature was elevated to 40ºC, EKM2 has shown 3.27 µm mycelial thickness followed by CKD (2.44 µm). The isolate M4 has shown a thickness of 2.47 µm which is smaller compared to EKM2. At the peak temperature of 41ºC, EKM2 has shown a highest mycelial thickness of 3.07 µm followed by M4 (2.65 µm) and CKD (2.59 µm) which are on par with each other (Plate 22).

Table 15. The shape of spores at different temperature levels

Isolates	35ºC	36ºC	37ºC	38ºC	39ºC	40ºC	41ºC
CKD	Oblong, oval, and dumbbell-shaped	Oval, elongated with thick walls	Elongated and oblong shaped	Elongated dumbbell-shaped with thick walls	Small oblong shaped and shrunken	Oval shrunken spores with small size	Very small round shaped with thick walls
EKM2	Elongate, oval, segmentation at both edges	Oval to a round shape, thick walls	Long oval shaped with little shrinkage	Long spores with little shrinkage	Elongate to round with small size and little shrinkage	Abrupt shape with small size and thick walls	Small narrow shaped which are shrunken
M4	Long, oval, thick-walled spores	Dumbbell shaped with thick walls	Large oval shaped with thick walls	Large, oval thick walled	-	-	-

*Mean of three replications

The results revealed that EKM2 is more thermotolerant with highest mycelial thickness at all temperature levels. The isolate M4 has shown low mycelial thickness compared to EKM2 and CKD except at 41ºC. From the results, it is clear that isolate EKM2 is superior to other isolates, CKD and M4.

4.7.6. Maintaining the thermotolerant isolates for six generations

The selected thermotolerant isolate EKM2 was grown at 41ºC continuously for up to six generations to confirm its temperature tolerance. The isolate EKM2 has shown considerable amount of growth and mycelial thickness at all the six generation with higher sporulation. The thermotolerant isolate EKM2 after six generations were used for the bioefficasy testing on *Galleria mellonella*and biochemical analysis.

Table 16. Effect of temperature on mycelial thickness of *Metarhizium anisopliae* isolates

Mean mycelial thickness at different temperature levels (µm)									
Isolates	**Control (28±2ºC)**	**35ºC**	**36ºC**	**37ºC**	**38ºC**	**39ºC**	**40ºC**	**41ºC**	**Mean**
CKD	6.647^{b}	5.247^{c}	3.580^{fg}	4.423^{d}	3.660^{fg}	3.450^{fgh}	2.393^{j}	2.597^{ij}	4.000^{b}
EKM2	7.297^{a}	7.160^{a}	6.490^{b}	3.513^{fgh}	4.333^{de}	3.877^{ef}	3.270^{gh}	3.070^{hi}	4.876^{a}
M4	5.123^{c}	3.687^{fg}	3.533f^{gh}	2.320^{j}	2.757^{ij}	2.690^{ij}	2.477^{j}	2.650^{ij}	3.155^{c}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

4.8. Inducing drought tolerance in *Metarhizium anisopliae* isolates

Inducing drought tolerance to *M. anisopliae* isolates was done at increasing levels of Polyethylene Glycol (PEG) concentrations starting from 25 per cent and continued up to 41per cent. The treatments started from 25 per cent of PEG Concentration and continued upto 35 % as a broad range. After wards the treatments are made narrow from 37 to 41 per cent. The mycelial weight of the isolates, size and shape of the spores, mycelial thickness, spore germination, and spore count at each level of PEG concentration was recorded. The results of the experiment are depicted below.

4.8.1. Effect of induced drought on mycelial weight of *Metarhizium anisopliae* isolates

The mycelial weight of the isolates grown in PEG-amended PD broth was measured after 10 days of inoculation at room temperature (28±2^{0}C). The results of the experiment are depicted in Table 17.

Table 17. Effect of drought on mycelial weight of *Metarhizium anisopliae* isolates

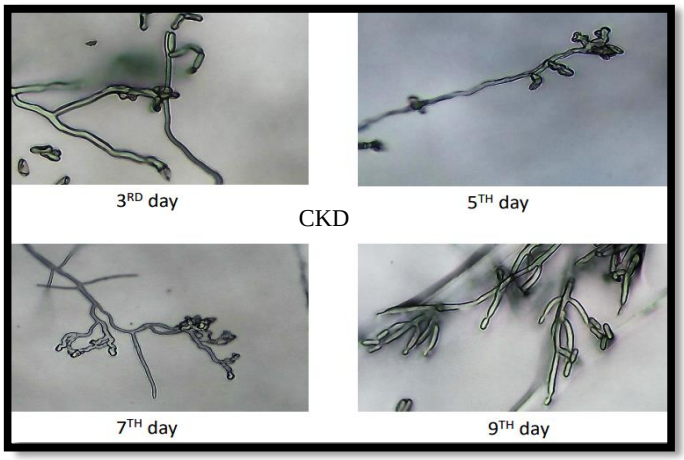
Mean mycelia weight at different concentrations of Polyethylene Glycol (PEG)(g/100ml)								
Isolates	**Control**	**25%**	**30%**	**35%**	**37%**	**38%**	**39%**	**40%**
CKD	3.455^{b}	3.400^{b}	3.365^{b}	2.850^{cd}	1.192^{f}	0.952^{fg}	0.858^{gh}	0.623^{hig}
EKM2	10.206^{a}	2.807^{cd}	2.890^{b}	2.623^{cd}	1.751^{e}	0.656^{ghij}	0.513^{ij}	0.387^{j}
M4	2.549^{d}	1.866^{d}	1.222^{f}	0.400^{j}	0.808^{ghi}	0.000^{k}	0.000^{k}	0.000^{k}

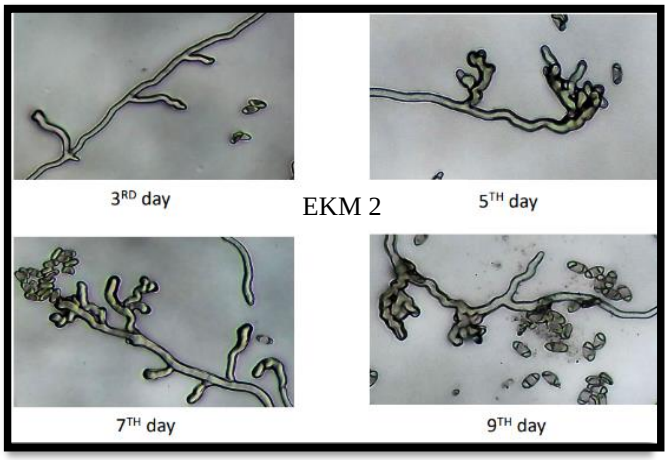
*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

The lowest concentration of PEG at which the experiment was started was 25 %. At 25 % of PEG concentration, the highest mycelial weight was observed in the isolate CKD (3.40 g) followed by EKM 2 (2.80g). The lowest mycelial weight at the same concentration was observed in isolate M4 (1.86g) (Plate 23). At 30 % of PEG concentration, CKD showed a mycelial weight of 3.36g followed by EKM2 which showed a weight of 2.89g. M4 has shown the lowest mycelial weight at 30 % of PEG concentration (Plate 24). At 35% PEG, the highest mycelial weight was shown by CKD (2.85g) followed by EKM2 (2.62g) which were on par with each other. The lowest weight was recorded by M4 (0.4 g) at the same concentration (Plate 24). At 37 % of PEG concentration, EKM2 ranked first with a mycelial weight of 1.75 g followed by CKD with a weight of 1.19 g. The isolate, M4 was the one with the lowest recorded mycelial weight (0.80 g) (Plate 25).

When the PEG concentration was increased to 38 per cent, CKD recorded a higher mycelial weight of 0.95 g followed by EKM2 with 0.65 g of mycelial weight. The isolate M4 has not shown any growth at 38 per cent of PEG concentration (Plate 25). When the PEG concentration was enhanced to 39 per cent, CKD has recorded a higher mycelial biomass of 0.85 g followed by EKM2 with a weight of 0.51g (Plate 26). The highest concentration of PEG at which isolates have grown was 40 per cent. At 40 per cent of the PEG concentration, CKD has shown a mycelial weight of 0.62 g followed by EKM2 with a weight of 0.38 g (Plate 26). At 41 % of PEG concentration, none of the isolates had shown any growth and the experiment was stopped at this level. The sporulation of the isolates at all levels of PEG concentration was observed (Plate 28). The isolate CKD has recorded the highest sporulation at all levels of PEG concentration (Table 18). The isolate EKM2 has also shown moderate sporulation at all levels of PEG concentration. The isolate M4 recorded least or no sporulation at all PEG levels.

From the results, it is clear that isolate CKD has shown high sporulation (Plate 27) and higher mycelial weight at all levels of PEG concentration ranging between 0.623 g to 3.365 g. The isolate EKM2 showed a mycelial weight ranging between 0.380 g to 2. 890 g with moderate sporulation. The lowest weight of mycelia was observed in isolate M4 with very low sporulation (Plate 27) at all levels of PEG concentration and which did not show any growth after 37% of PEG. Hence, the





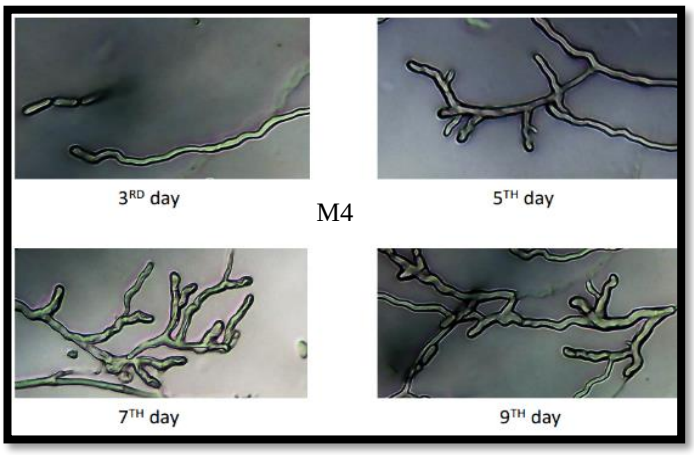
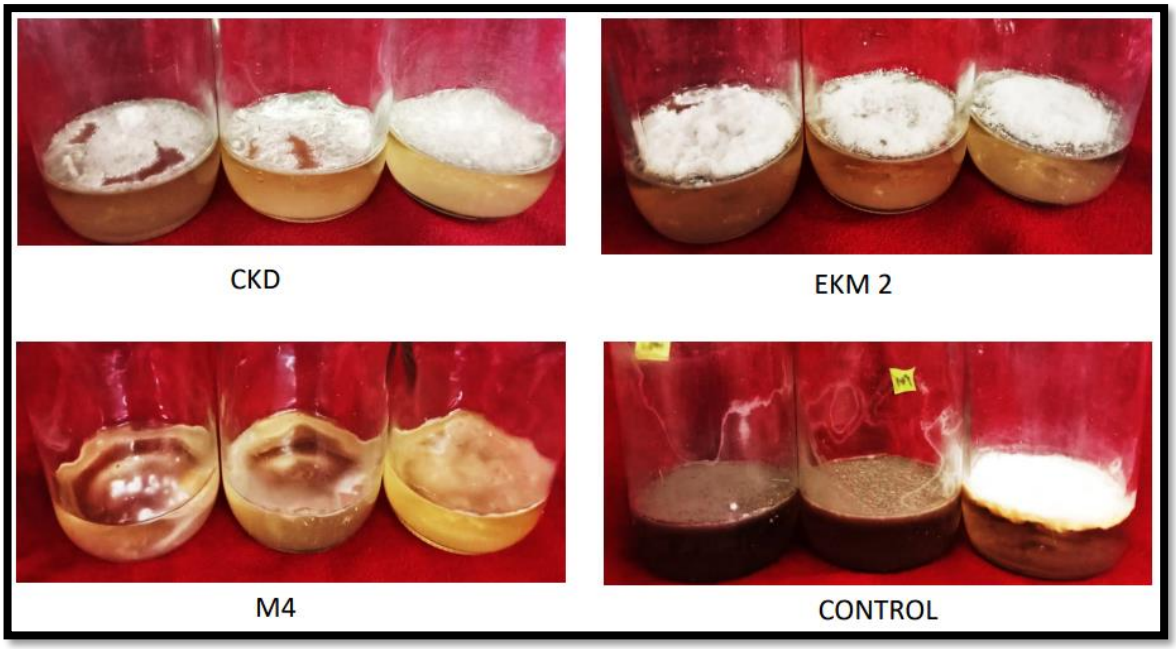
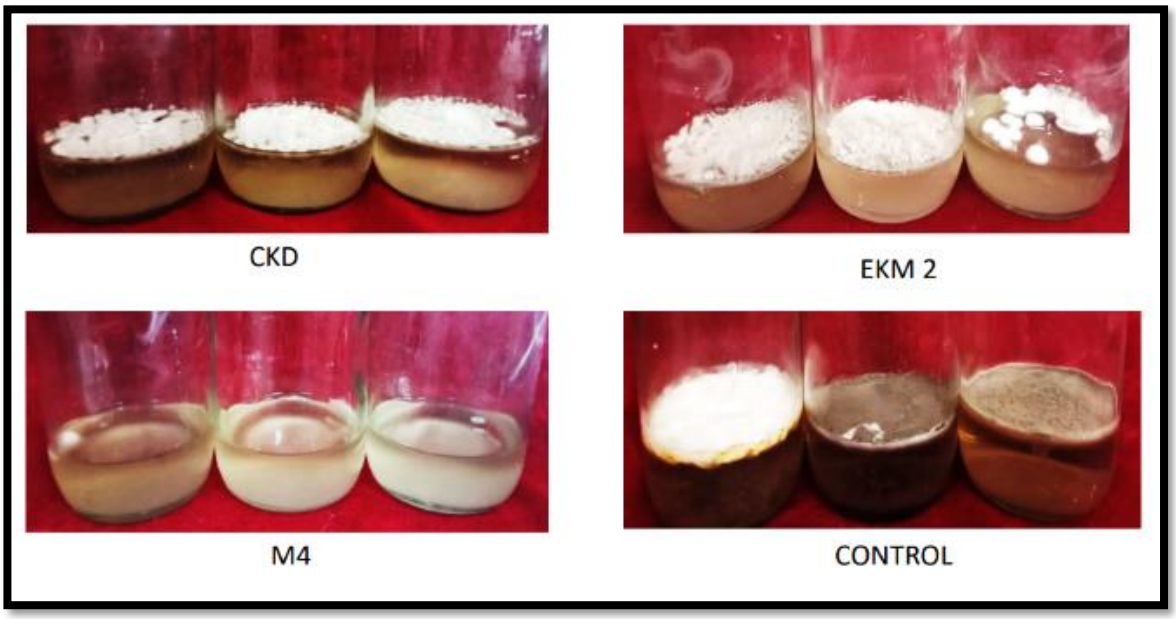


Plate 23. Effect of induced drought (25% PEG) on growth, sporulation, spore size and mycelial thickness of *Metarhizium anisopliae* isolates

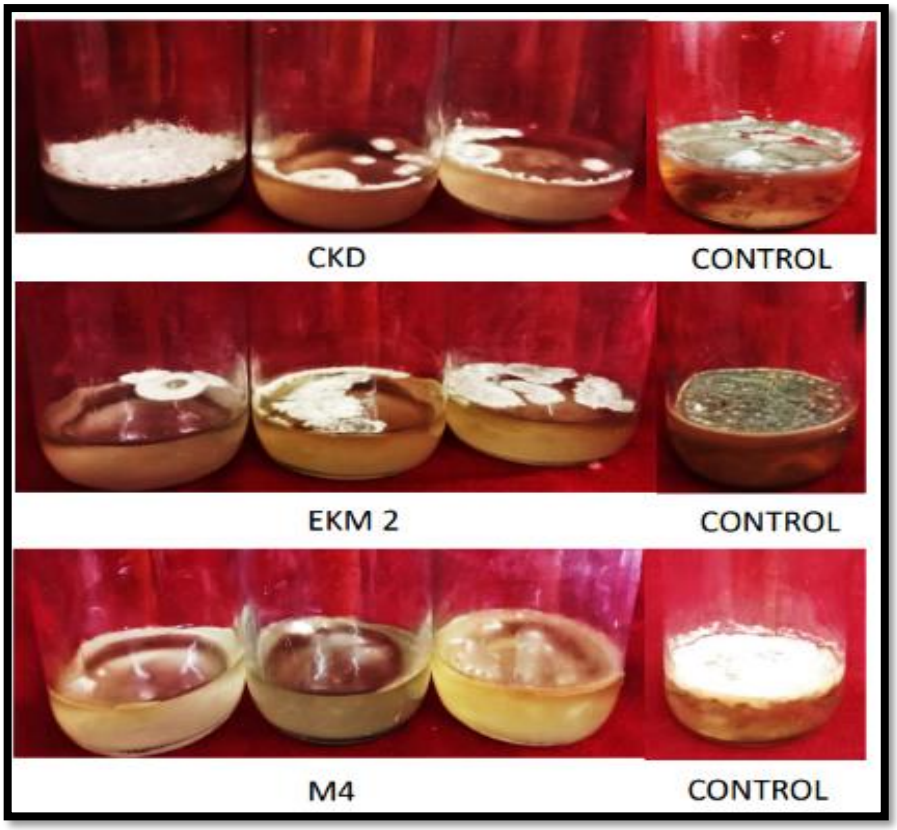


30 % PEG

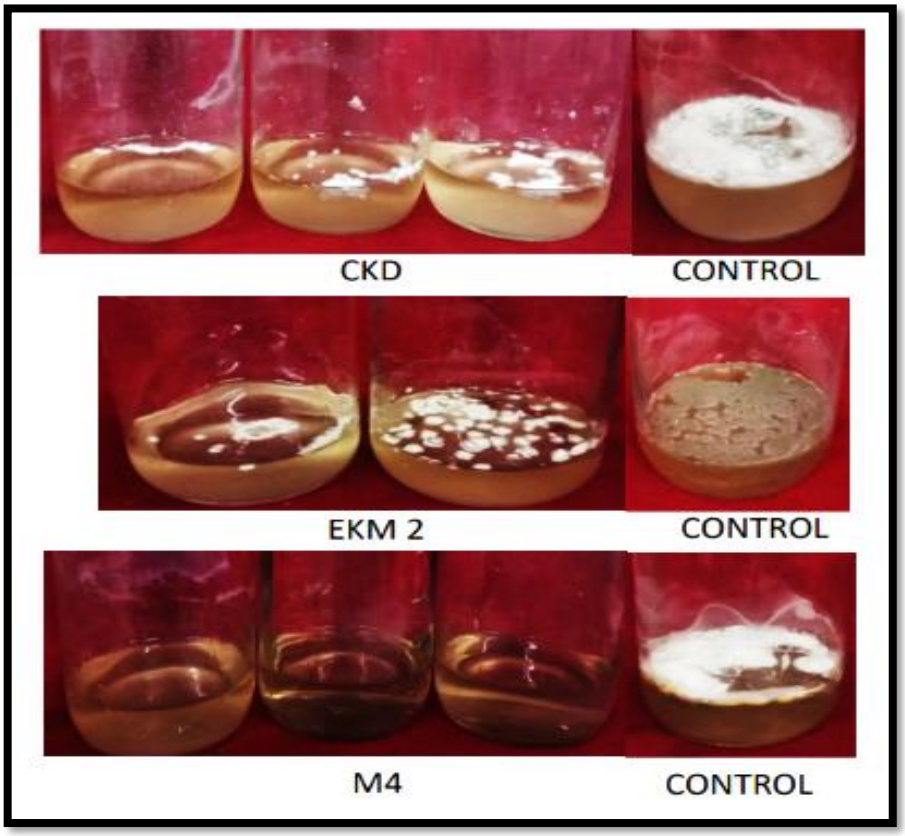


35 % PEG

Plate 24. Effect of induced drought on growth and sporulation of *Metarhizium anisopliae* isolates

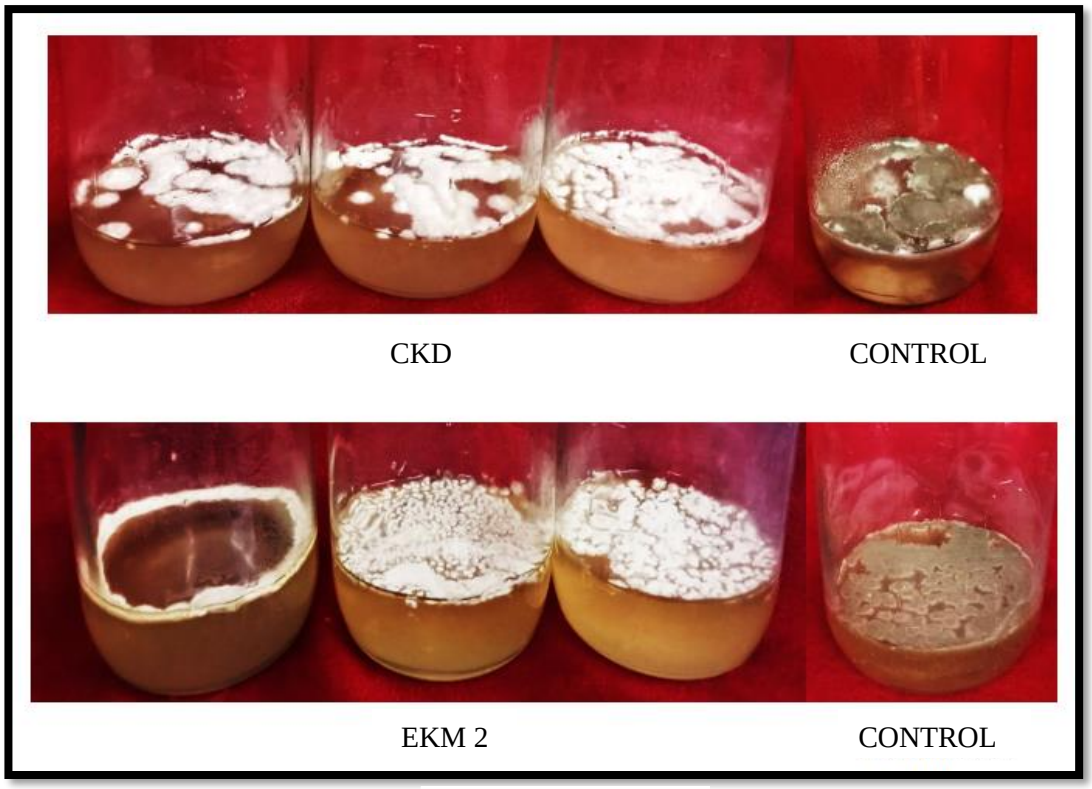


37 % PEG

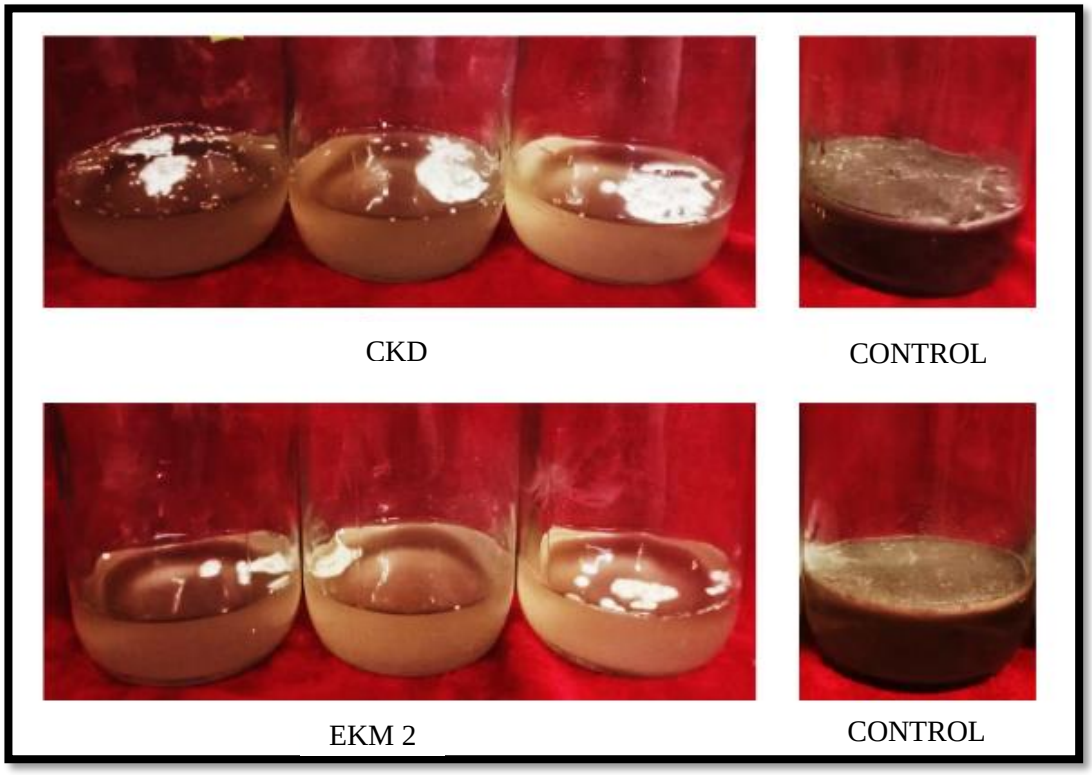


38 % PEG

Plate 25. Effect of induced drought on growth and sporulation of *Metarhizium anisopliae* isolates

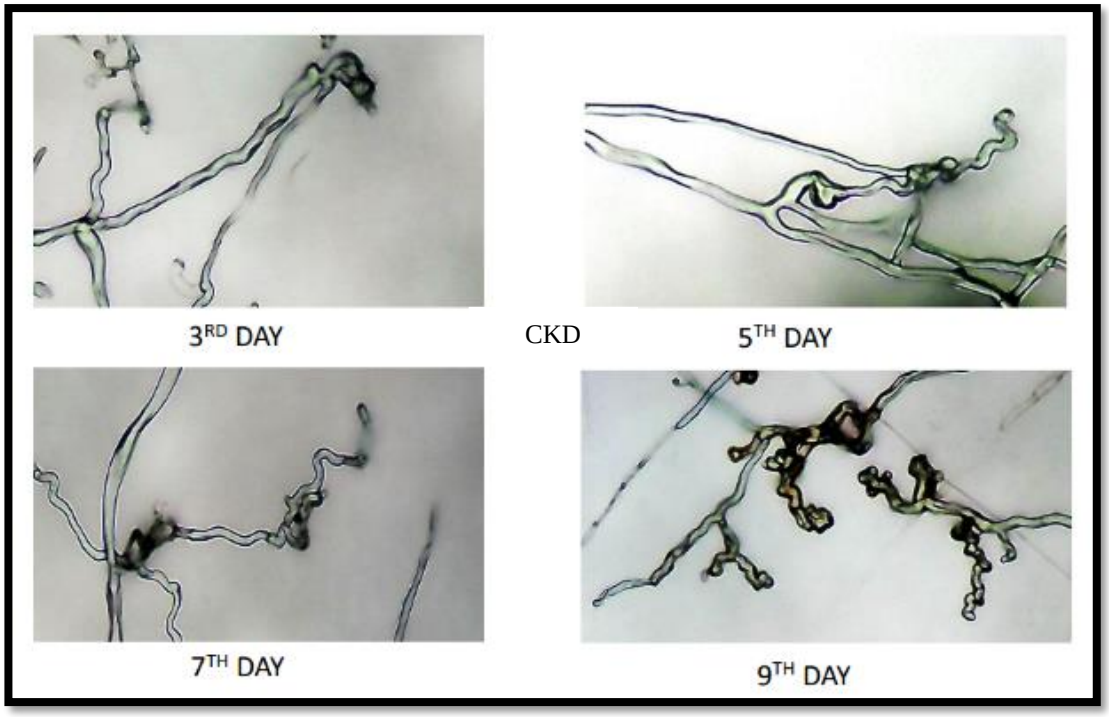


39 % PEG



40 % PEG

Plate 26. Effect of induced drought on growth and sporulation of *Metarhizium anisopliae* isolates



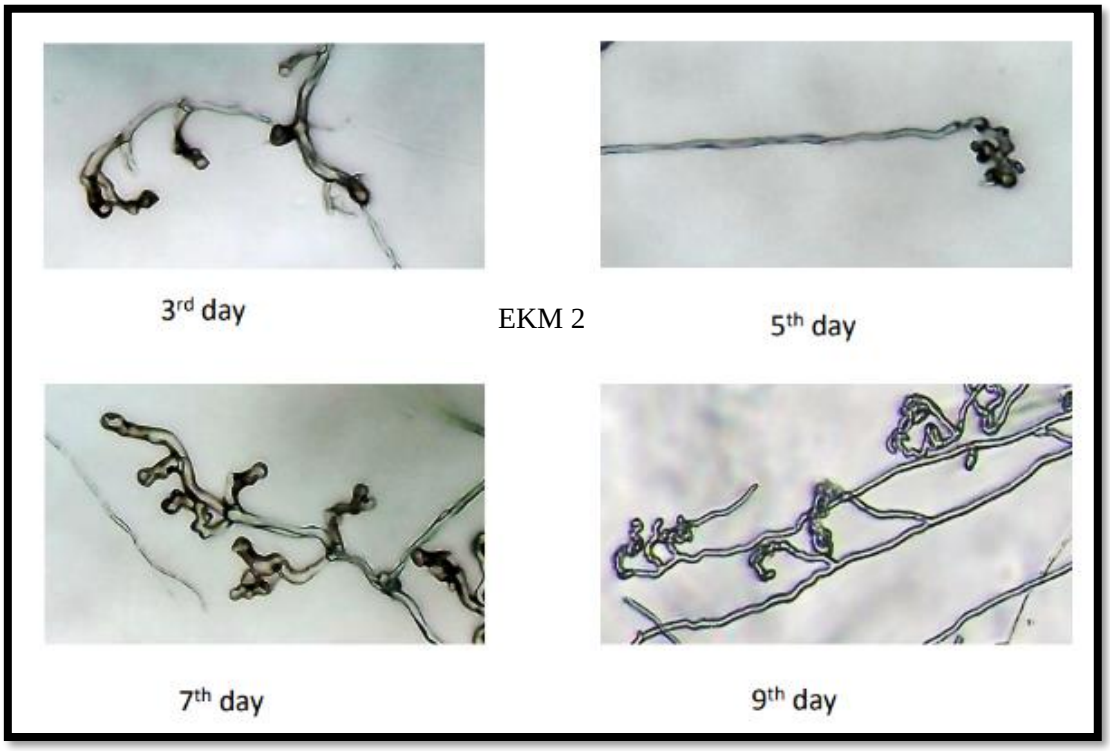
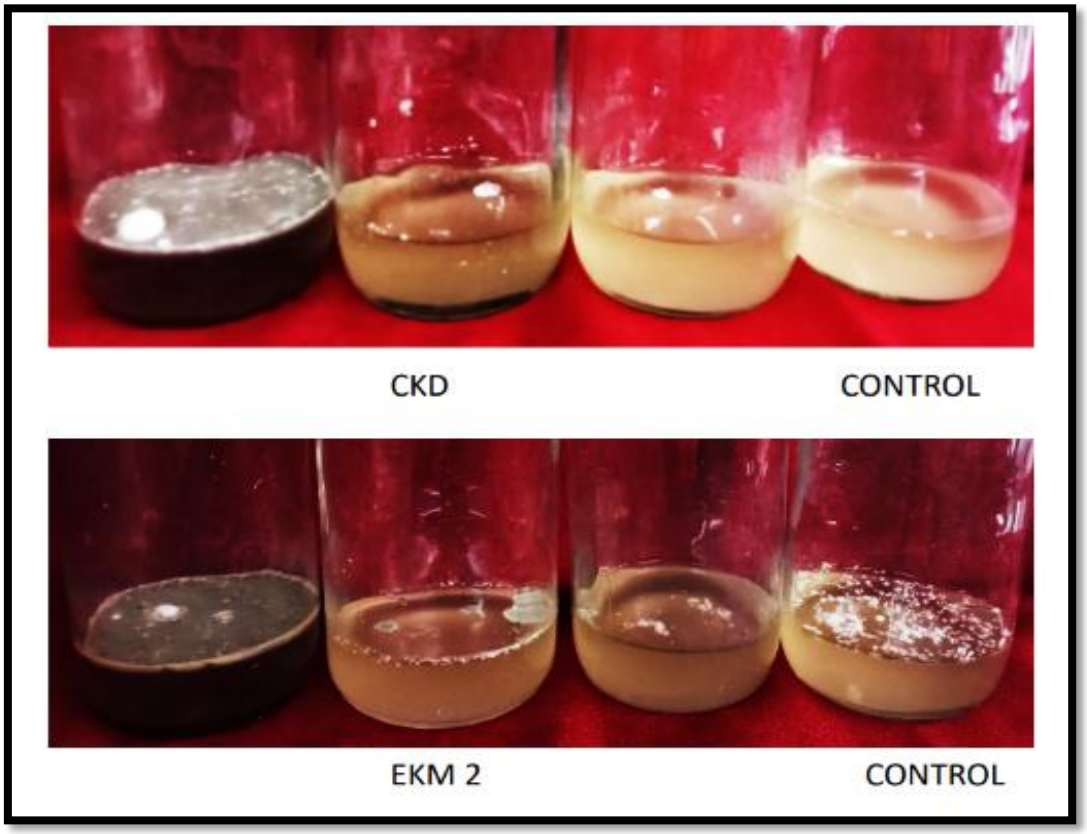


Plate 27. Effect of induced drought (40% PEG) on spore size and mycelial thickness of *Metarhizium anisopliae* isolates



41 % PEG

Plate 28. Effect of induced drought on growth and sporulationof *Metarhizium anisopliae* isolates

isolate CKD which proved to be superior among the three identified as tolerant to drought. M4 has been identified as the drought susceptible isolate.

Table 18. Effect of induced drought on sporulation *of Metarhizium anisopliae* isolates

solate	Sporulation at different levels of PEG						
	25%	30%	35%	37%	38%	39%	40%
CKD	+++	+++	+++	+++	+++	++	+
EKM2	+++	++	+++	++	++	+	-
M4	+	+	+	+	-	-	-

+++: High sporulation, ++: Medium sporulation, +: Sparse sporulation, -: No sporulation

4.8.2. Effect of induced drought on spore germination of *Metarhizium anisopliae* isolates

The spore germination of isolates at different levels of PEG concentration was recorded by counting the number of colonies produced at 3rd dilution. The colony-forming units (CFU/ml) were calculated and the results of the experiment are given in Table 19.

Table. 19. Effect of induced drought on spore germination of *Metarhizium anisopliae* isolates

Mean spore germination at different concentrations of Polyethylene Glycol (cfu/ml)*								
Isolates	**Control**	**25%**	**30%**	**35%**	**37%**	**38%**	**39%**	**40%**
CKD	122.00^{a}	92.00^{b}	86.667^{bc}	39.000^{f}	21.667^{g}	9.333^{i}	2.000^{j}	4.667^{j}
EKM2	92.000^{b}	82.233^{cd}	87.667^{bc}	36.667^{f}	16.333^{gh}	4.333^{ij}	1.333^{ij}	0.667^{j}
M4	79.333^{d}	59.667^{e}	61.333^{e}	15.667^{h}	2.00^{j}	0.000^{j}	0.000^{j}	0.000^{j}

* Number of colonies X 10^3/ml (CFU/ ml at 3rd dilution)***** Mean of three observations

The highest number of colonies at 25 per cent of PEG concentration was observed in the isolate CKD (92 X 10^3 CFU/ ml) followed by EKM2 (82 X 10^3 CFU/ ml). The least number of colonies was observed in isolate M4 (59 X 10^3 CFU/ ml).

At 30% of PEG concentration, EKM2 recorded the highest CFU/ml (87 X 10^3colonies/ ml) followed by CKD (86 X 10^3colonies/ ml), and the least CFU/ml was observed in M4. At 35% of PEG concentration, CKD ranked first with a higher number of colonies *ie*, 39 X10^3colonies/ ml, followed by EKM2 with 36 X 10^3 CFU / ml. When the PEG concentration was increased to 37 per cent, CKD has shown a colony count of of 21 X 10^3 CFU/ ml followed by EKM2 with 16 X 10^3 CFU/ ml. The isolate M4 has shown the least number of fungal colonies (2 X 10^3 CFU/ ml) at 37 per cent PEG. At enhanced PEG concentration of 38 per cent, CKD ranked first with 9 X 10^3 CFU/ ml followed by EKM2 with 4.33 10^3 CFU/ ml. When the isolates were exposed to higher PEG concentration of 38 per cent, M4 has stopped the growth and hence did not produce any colonies. The number of colonies/ml produced at 39 and 40 per cent was very low compared to previous treatments. At 39 per cent of PEG, CKD produced 2 X 10^3 CFU/ ml followed by EKM2 which produced 1 X 10^3CFU/ ml. At the peak PEG concentration of 40 per cent, CKD showed a CFU of 4 X 10^3colonies/ ml and EKM2 resulted in 0.66 X 10^3 CFU/ ml.

From the results, it is evident that a higher CFU/ml was observed for the isolate CKD at all levels of PEG concentration, followed by EKM2. The least CFU/ml was recorded in isolate M4. Hence, CKD was identified to be tolerant to drought and M4 is found as susceptible. The isolate EKM2 is found to be moderately tolerant to drought.

4.8.3. Effect of induced drought on spore count of *Metarhizium anisopliae* isolates

The spore count in one ml of PDB at all levels of PEG concentration was recorded using a hemocytometer. The results of the experiment are depicted in Table. 20

Table 20. Effect of induced drought on spore count of *Metarhizium anisopliae* isolates

Mean spore count at different concentrations of Polyethylene Glycol (spores/ml)*								
Isolates	**Control**	**25%**	**30%**	**35%**	**37%**	**38%**	**39%**	**40%**
CKD	24.900^{a}	15.000^{b}	13.333^{c}	0.567^{i}	0.617^{i}	0.400^{i}	0.333^{i}	0.323^{i}
EKM2	11.033^{b}	6.300^{f}	4.250^{g}	0.467^{i}	0.500^{j}	0.400^{i}	0.467^{i}	0.237^{j}
M4	8.200^{e}	1.733^{h}	1.783^{h}	0.400^{i}	0.243^{i}	0.000^{j}	0.000^{j}	0.000^{j}

*Spore count X 10^7 /ml**Mean of three observations

The spore counts at all levels of PEG concentrations were expressed as number of spores/ml. At 25 per cent of PEG, isolate CKD has shown a spore count of 15X 10^7 /ml followed by EKM2 with 6.3 X 10^7 /ml. The isolate M4 was the one with the lowest spore count of 1.73X 10^7 /ml. When the PEG concentration was increased to 30 %, CKD has shown a spore count of 13.33 × 10^7 /ml, which is significantly higher than other isolates. At the same concentration, EKM2 has ranked second with 4.2X 10^7 /ml followed by M4 with the lowest spore count of 1.78 ×10^7 /ml. At 35 per cent of PEG, the spore count of all the isolates was significantly low. At the same concentration, CKD was recorded higher spore count of 0.56X 10^7 /ml followed by EKM2 with 0.46X 10^7 /ml. The lowest spore count was recorded in isolate M4 at 0.40X 10^7 /ml. When the PEG concentration was increased to 37%, the highest spore count was observed in the isolate CKD (0.61 X 10^7 /ml) followed by EKM2 (0.50X 10^7 /ml) and the least spore count was recorded in isolate M4 (0.24X 10^7 /ml). At 38 % PEG, the isolates CKD and EKM2 recorded a spore count of 0.40 X 10^7 /ml which were on par with each other. The isolate, M4 did not show any growth at 38% PEG. The spore count of EKM2 was higher (0.46) followed by CKD (0.33X 10^7 /ml) which were on par with each other at 39 per cent PEG concentration. Maximum PEG concentration of 40 % resulted in higher spore count in CKD (0.323X 10^7 /ml) followed by EKM2 (0.23X 10^7 /ml).

The isolate CKD has recorded a higher spore count/ ml in PD broth at all PEG concentrations. Hence, CKD is selected as a drought-tolerant isolate. The isolate EKM2 with a moderate number of spores/ ml was identified to be moderately tolerant to drought. The isolate M4 was the one with the lowest spore count at all levels of PEG and was found as the susceptible isolate.

4.8.4. Effect of induced drought on size and shape of spores of *Metarhizium anisopliae* isolates

The spore size was measured in micrometers using the computer software Radical. The shape of the spores was also observed using an image analysing compound microscope. The results of the experiment are given in Table 21.

At 25 per cent PEG concentration, EKM2 has shown a higher spore length of 9.22 µm followed by CKD with a length of 8.32 µm. The lowest spore size was observed in the isolate M4 (6.43 µm) (Plate 23). At the next incremental level of PEG (30 %), EKM2 was the one with highest spore length (8.57 µm) followed by CKD

(8.26 µm) which were on par with each other. The lowest spore length at the same concentration was observed in the isolate M4 (6.38 µm). From 35 per cent PEG onwards CKD has recorded the highest spore length. At 35 per cent of PEG, CKD showed a spore length of 5.26µm followed by M4 (4.49 µm). The lowest spore length was found in EKM2 with 3.93 µm. At a higher PEG concentration (37 % PEG), CKD recorded higher spore length (3.13 µm) followed by M4 (4.49 µm). The lower length of spore was observed in isolate EKM2 (2.36 µm). When the PEG concentration was increased to 38 per cent, CKD has shown a spore length of 3.15 µm followed by EKM2 (2.45 µm). From 38 per cent PEG onwards, M4 did not showed any growth.

Table 21. Effect of induced drought on Spore size of *Metarhizium anisopliae* isolates

Mean spore size at different concentrations of Polyethylene Glycol (µm)								
Isolates	**Control**	**25%**	**30%**	**35%**	**37%**	**38%**	**39%**	**40%**
CKD	12.683^{a}	8.320^{c}	8.263^{c}	5.263^{e}	3.133^{h}	3.150^{h}	2.687^{hi}	2.277^{i}
EKM2	13.013^{a}	9.220^{b}	8.573^{c}	3.930^{g}	2.360^{i}	2.450^{i}	2.460^{i}	2.390^{i}
M4	6.970^{d}	6.433^{d}	6.387^{d}	4.493^{g}	4.493^{ef}	0.000^{i}	0.000^{i}	0.000^{i}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

Higher PEG concentration of 39 per cent, resulted in a higher spore length of 2.68 µm in CKD and was followed by EKM2 with a length of 2.46 µm. The isolates when exposed to a peak PEG concentration (40 %), EKM2 has recorded the highest spore length (2.39 µm) followed by CKD (2.27 µm) which were on par with each other (Plate 27). The shape of the spores at different levels of PEG was also recorded and the results are depicted in table 22.

At 25 per cent of PEG, CKD has shown elongated, oval, large spores while EKM2 showed long, oval spores with segmentation at both edges. The isolate M4 with long, oval thick-walled spores were observed at 25 per cent PEG concentration (Plate 23). At a higher PEG concentration of 30 per cent, CKD has shown oval, elongated spores with a bigger size and EKM2 has shown oval to round-shaped spores with thick walls. The spores of the isolate M4 was dumbbell-shaped with thick walls. The isolate CKD was recorded with narrow to oblong-shaped shrunken spores at 35 per cent of PEG. At the same PEG concentration, EKM2 was with long oval-

shaped spores with little shrinkage and M4 was with large oval to round-shaped thick-walled spores. Long, oval with thick-walled spores were observed in the isolate CKD at 37 per cent of PEG and long oval-shaped spores with little shrinkage were observed in EKM2. The isolate M4 has shown large, oval, thick-walled spores at 37 per cent PEG. At a higher PEG concentration of 38 per cent, CKD has shown small, long, oval-shaped, and shrivelled spores and EKM2 has shown oval to round spores with small size and shrinkage. The isolate M4 was with large oval spores with thick walls. When the PEG concentration was increased 39 per cent, CKD has recorded a spore shape of oval, shrunken with small size and EKM2 was with abrupt shaped spores of small size. At a higher droght level with 40 per cent of PEG concentration, CKD has shown very small round shaped spores with thick walls and EKM2 has shown small narrow shaped shrunken spores (Plate 27).

The results are showing that CKD was the one that has shown a higher spore length at almost all levels of PEG. Hence, CKD was identified as the drought-tolerant isolate. The isolateM4 with the lowest spore size at all levels of PEG was proved to be susceptible. EKM2 was moderately tolerant to drought.

Table 22. The shape of spores at different concentrations of PEG

Isolates	25%	30%	35%	37%	38%	39%	40%
CKD	Elongate, oval, large spores	Oval, elongated with a bigger size	Narrow to oblong-shapedshrunken spores	Long, oval with thick walls	Small, long, oval shaped and shrinked	Oval shrunken spores with small size	Very small round shaped with thick walls
EKM2	Long, oval, segmentation at both edges	Oval to a round shape, thick walls	Long oval shaped with little shrinkage	Long, dumbbell shaped, spores with shrinkage	Oval to round with small size and shrinkage	Abrupt shape with small size	Small narrow shaped which are shrunken
M4	Long, oval, thick-walled spores	Dumbbell shaped with thick walls	Large oval to round shaped thick-walled	Large, oval thick walled	-	-	-

*****Mean of three observations

4.8.5. Effect of induced drought on the mycelial thickness of *Metarhizium anisopliae* isolates

The mycelial thickness at all levels of PEG concentrations was measured by observing through a compound microscope with an image analyser. The thickness was measured in micrometers using the computer software Radical. The results are depicted in table 23.

Initially at 25 per cent PEG concentration, the highest thickness of mycelia was observed in the isolate CKD (4.79 µm) followed by EKM2 (3.91 µm). The least thickness of mycelia was observed in the isolate M4 (2.72 µm) (Plate 23). At 30 per cent of PEG, the highest thickness was observed in the isolate CKD (4.58 µm) followed by EKM2 (3.96 µm). The mycelial thickness of M4 at 30 per cent was 2.51µm. When the PEG concentration was increased to 35 per cent, EKM 2 has recorded a higher mycelial thickness of 3.16 µm followed by CKD with 2.71 µm. The isolate M4 has shown 2.59 µm thickness at 35 per cent of PEG. At a higher level of 37 per cent PEG, CKD has shown a mycelial thickness of 2.85 µm followed by EKM2 (2.43 µm) and M4 has shown a thickness of 2.54 µm. When the isolates were exposed to 38 per cent PEG, highest mycelial thickness was observed in the isolate CKD (3.77 µm) followed by EKM2 (2.47 µm) which were on par with each other. At 39 per cent, the isolate CKD has recorded a mycelial thickness of 2.58µm and the isolate EKM2 has recorded a 3.45 µm thickness. Peak PEG concentration of 40 per cent resulted in highest mycelial thickening in the isolate EKM2 was 2.72 µm thickness and it was followed by CKD (2.53 µm) (Plate 27).

The results indicate that CKD has recorded a higher mycelial thickness at all temperature levels. The isolate M 4 has shown low mycelial thickness compared to CKD and EKM2. From the results, it is clear that isolate CKD is superior to other isolatesEKM2 and M4.

Table 23. Effect of induced drought on the mycelial thickness of *Metarhizium anisopliae* isolates

Mean mycelial thickness at different concentrations of Polyethylene Glycol (µm)								
Isolates	**Control**	**25%**	**30%**	**35%**	**37%**	**38%**	**39%**	**40%**
CKD	6.647^{b}	4.797^{cd}	4.587^{d}	2.713^{ghi}	2.853^{ghi}	3.777^{i}	3.240^{fg}	2.530^{i}
EKM2	7.297^{a}	3.910^{i}	3.960^{e}	3.167^{gh}	2.433^{i}	2.477^{i}	2.583^{i}	2.723^{hi}
M4	5.123^{d}	2.727^{hi}	2.510^{i}	2.590^{i}	2.543^{i}	0.000^{i}	0.000^{i}	0.000^{i}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

4.8.6. Maintaining the drought tolerant isolates for six generations

The selected drought tolerant isolate CKD was grown at 40 per cent PEG continuously for six generations to confirm its drought tolerance. The isolate CKD has shown considerable amount of growth and mycelial thickness at all the six generation with higher sporulation. The drought tolerant isolate CKD after six generations were used for the bioefficasy testing on *G. mellonella* and biochemical analysis.

4.9. *In vitro* screening of *Metarhizium anisopliae* Sorokin. isolates for biocontrol efficacy

The biocontrol efficacy of the selected tolerant isolates was carried out against *G. mellonella* larvae at doses of 10^5 to $10^{9.}$ The mortality of 3rd instar larvae of wax moth at 24 h intervals up to 10 days and LT_{50} were worked out (Plate 29). The results of the experiments are furnished in tables 24 and 25.

Table 24. Mortality of *Galleria mellonella larvae* (%) caused by *Metarhizium anisopliae* isolates

Isolate	Dose	3rd day	5th day	7th day	9th day
CKD	$1X10^5$	-	-	30	36.66
	$1X10^6$	-	-	50	80
	$1X10^7$	-	26.66	63.33	90
	$1X10^8$	-	40	80	96.66
	$1X10^9$	-	73.33	86.66	100
EKM2	$1X10^5$	-	-	36.66	66.66
	$1X10^6$	-	-	60	80
	$1X10^7$	-	-	73.33	86.66
	$1X10^8$	-	26.66	86.66	100
	$1X10^9$	-	33.33	90	100
M4	$1X10^5$	-	-	26.66	70
	$1X10^6$	-	-	43.33	76.66
	$1X10^7$	-	-	53.33	80
	$1X10^8$	-	20	66.66	83.33
	$1X10^9$	-	26.66	70	86.66

4.9.1. Mortality of *Galleria mellonella larvae* (%) caused by *Metarhizium anisopliae* isolates

The effect of selected tolerant isolates of *M. anisopliae*, CKD (drought tolerant isolate), and EKM2 (temperature tolerant isolate) was evaluated using the contact toxicity bioassay method. The bioefficacy of the susceptible isolate M4 was also carried out for comparison. The number of insects killed at different concentrations of spore suspension was observed at 24 h intervals up to 10 days (Plate 29). The results are given in Table 24.

Three days after treatment, none of the isolates has shown mortality of the larvae at all concentrations. All the isolates have shown mortality from the 5^{th} day onwards (Plate 30). On the 5^{th} day, the isolate CKD has shown mortality at 10^7 to 10^9 spores/ml, ranging from 26.66 to 73.33 per cent. The isolate, EKM2 has shown a mortality of 26.66 per cent at 10^8 spores/ml and 33.33 per cent at 10^9 spores/ml of concentrations on the 5^{th} day. The isolate, M4 has also shown a mortality of 20 per cent and 26.66 per cent at $10^8$and 10^9concentrations respectively. Higher mortality was observed at 10^9 spores/ml in all the isolates on the 5^{th} day. Only CKD has shown mortality at 10^7spores/ml on the 5^{th} day (26.66%). After 5 days of treatment, CKD has shown a higher mortality rate compared to EKM2 and M4 (Plate 30).

After 7 days of treatment, all the concentrations of all isolates have shown mortality (Plate 30). On the seventh day at spore concentration of 10^5 spores/ml, CKD has shown a mortality of 30 per cent, and EKM2 has shown a mortality of 36.66 per cent. M4 has shown 26.66 per cent mortality at 10^5 spores/ml. At 10^6spores/ml, CKD and EKM2 have shown mortality of 50% and 60% respectively. The isolate M4 has shown 43.33 per cent mortality at the same concentration. When the concentration was increased to 10^7spores/ml, CKD has shown 63.33 per cent mortality and EKM2 has shown a higher mortality of 73.33 per cent. The lowest mortality rate of 43.33 per cent was observed in isolate M4. A higher mortality rate of 80, 86.66, and 66.66 per cent was shown by CKD, EKM2, and M4 respectively at 10^8 spores/ml. At the highest concentration of 10^9 spores/ml, all the isolates have shown a significantly higher mortality rate. CKD, EKM2, and M4 have shown a mortality rate of 86.66, 90, and 70 per cent respectively. On the 7^{th} day of treatment, EKM2 has shown a higher mortality rate compared to CKD and M4.

Nine days after treatment all isolates have shown a higher mortality rate. At 10^5spores/ml, CKD has shown 43.33 per cent mortality and EKM2 has shown 66.66 per cent mortality. The isolate, M4 has shown a mortality of 33.33 per cent at the same concentration. At 10^6spores/ml, CKD, EKM2, and M4 have shown mortality of 63.33, 73.33, and 53.33 per cent respectively. The isolate CKD has shown a higher mortality of 96.66 per cent at 10^8 spores/ml whereas EKM2 has shown cent per cent mortality at the same spore concentration. The isolate, M4 has shown a mortality of 83.33 per cent. At the highest dosage of 10^9 spores/ml, both CKD and EKM2 have shown cent per cent mortality each and M4 has shown 86.66 per cent mortality. At the 9^{th} day EKM2 has shown a higher mortality rate at all doses compared to CKD and M4 (Plate 31).

The results showed that higher mortality at all concentrations of spores was shown by EKM2 followed by CKD. $10^8$and 10^9 spores/ml dosages have shown a higher mortality rate followed by 10^7spores/ml at which moderate mortality was observed. At 10^5 and 10^6 spores/ml, the mortality was very low compared to higher doses.

Only EKM2 has shown cent per cent mortality at 10^8 and 10^9 spores/ml of dosage on the 9^{th} day of treatment. Hence, EKM2 has proven to be a superior isolate when compared to CKD. Isolate CKD has also shown a higher mortality rate compared to M4. The isolate M4 which was susceptible has also shown an appreciable rate of mortality.

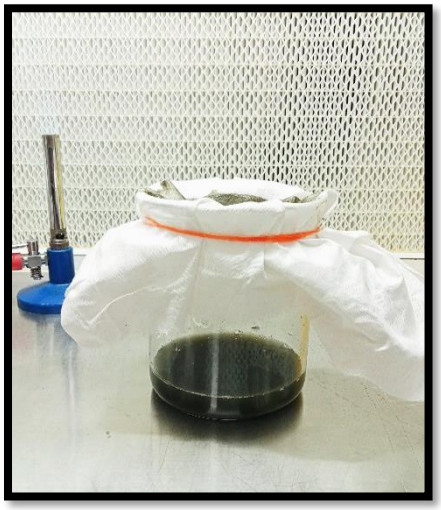
4.9.2. Dose – mortality responses of *Galleria mellonella* larvae

The lethal time (LT_{50} and LT_{90}) for mortality of Galleria larvae after treating with *M. anisopliae* isolates were calculated (Plate 30 and 31). The selected tolerant isolates and the susceptible isolate were used for treatment with a dosage of 10^5 to 10^9spores/ ml concentrations. The results of the experiment is given in Table 25.

At the lowest concentration of 1x 10^5spores/ml, the isolate EKM2 showed the shortest time to kill 50 per cent of the test insects (7.989 days) followed by CKD (8.806 days). The longest time was taken by the isolate M4 (9.46 days) for killing 50 per cent of the test population. The time required for killing 90 percentage of the test population was lowest in EKM2 (10.788 days) followed by CKD (13.887 days). The highest time was taken by M4 (14.097 days) for killing 90 per cent test population. At

i) Grinding 10 days old mycelial mat

ii) Filtering through muslin cloth



iii) Spore suspension of *Metarhizium anisoplaie*

iv) Serial dilution of the spore suspension

v) Application of 1 ml of spore suspension

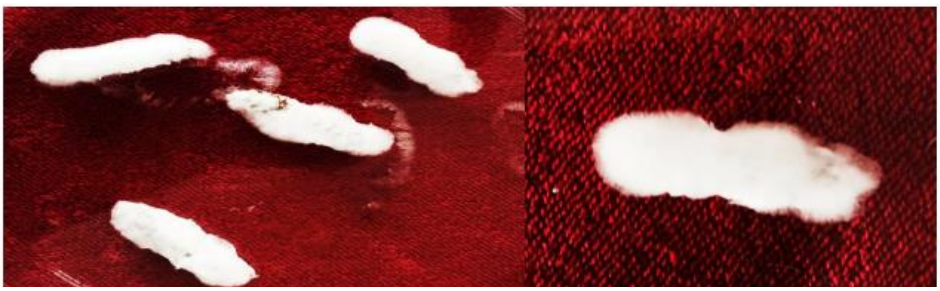
vi) Ten number of larvae maintained per replication

Plate 29. Evaluation of bioefficasy of *Metarhizium anisoplie* isolates against larvae of *Galleria mellonella*

CKD, EKM 2 and M4 infection on 20-day old larvae (5th day)

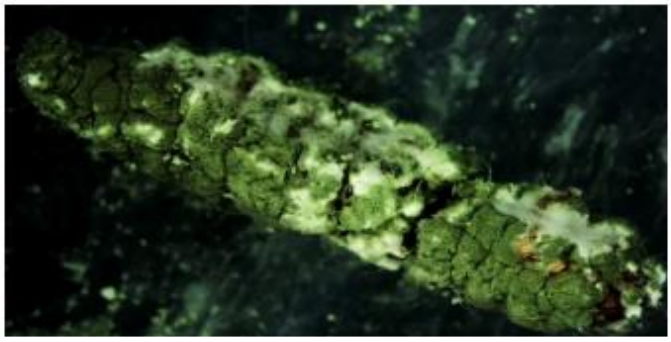
CKD infection on 20-day old larvae (7th day)

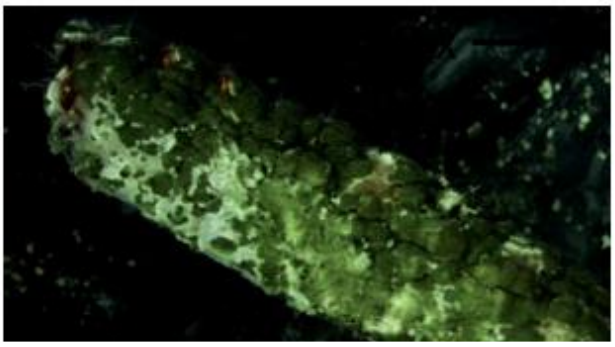
EKM 2 infection on 20-day old larvae (7th day)



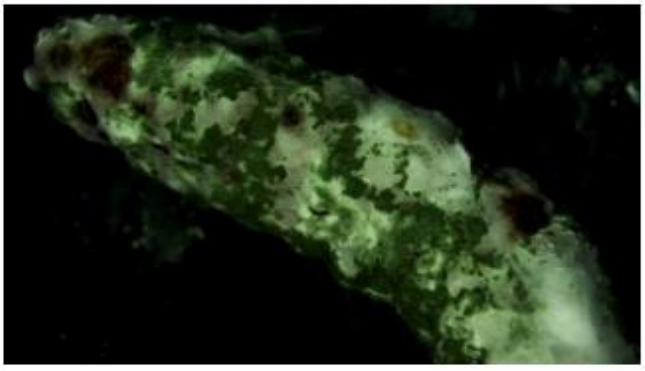
M4 infection on 20 day old larvae (7th day)

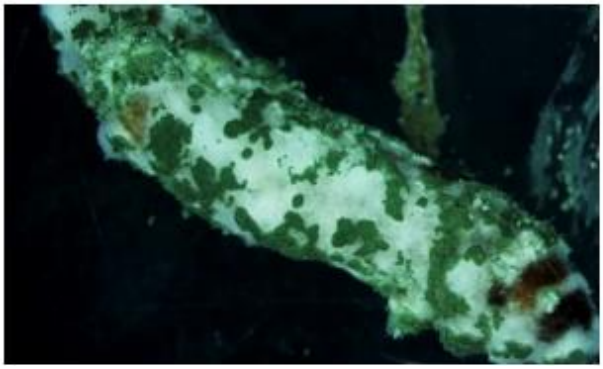
Plate 30. Infection of *Metarhizium anisopliae* isolates on 3rd instar larvae of *Galleria mellonella* 5 and 7 days after treatment



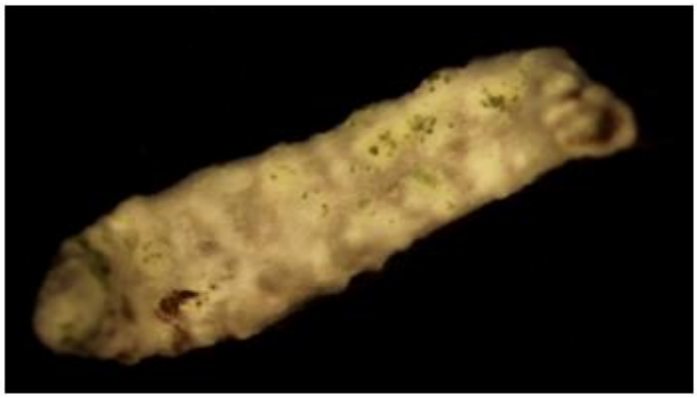


CKD infection on 20 day old larvae (10th day)





EKM 2 infection on 20 day old larvae (10th day)



M4 infection on 20 day old larvae (10th day)

Plate 31. Infection of *Metarhizium anisopliae* isolates on 3rd instar larvae of *Galleria mellonella* ten days after treatment

the lowest dosage of 1x 10^5spores/ml the isolate EKM2 has shown lower lethal time compared to other isolates followed by CKD.

At a dosage of 1x 10^6 spores/ml, EKM2 has shown the lowest LT_{50}value (7.125 days) followed by CKD (7.278 days). The highest LT_{50} was found in the isolate M4 (7.507 days). The LT_{90}value was also lower in the isolate EKM2 (9.466 days) followed by CKD (9.771 days) and the higher $LT_{90}$was found in the isolate M4. At the concentration of 1x 10^6spores/ml also EKM2 has taken few days for 50 per centage and 90 per cent mortality of the *Galleria* larvae.

At 1x 10^7 spores/ml of dosage, EKM2 has taken only 6.621 days for the 50 per cent mortality of the larvae followed by CKD which has taken 6.388 days. The highest time of 7.114 days was taken by the isolate M4. The LT_{90} value was also found lowest in EKM2 (8.488 days) followed by CKD (8.729 days). The higher LT_{90} value was observed in the isolate M4 which has taken 9.547 days to kill 90 per cent of the larvae. At 1x 10^7 spores/ml of dosage also EKM2 has recorded lowest LT_{50} and LT_{90}values.

At a concentration of 1x 10^8spores/ml, the shortest duration required for the 50 per cent mortality of the larvae was observed in the isolate EKM2 (5.414 days) followed by CKD (5.912 days). The highest LT_{50} value was found in the isolate M4 which has taken 6.390 days for killing 50 per cent of the test population. The LT_{50} value was found to be lower in all the isolates at this concentration compared to the lower dosages. LT_{90}values of the isolates EKM2, CKD and M4 at the dosage of 1x 10^8 spores/ml were 7.335 days, 8.033 days and 9.115 days respectively which is also lower compared to the previous dosages. EKM2 has shown lower LT_{50} and LT_{90}values at the concentration of 1x 10^8 spores/ml, followed by CKD.

The highest concentration of the isolates used for treatment was 1x 10^9 spores/ ml. At the highest dosage, the isolate CKD has shown a shorter LT_{50} value of 5.099 days, followed by EKM2 with LT_{50} value of 5.359 days. The highest LT_{50} value of 6.124 days was shown by the isolate M4. The LT_{90} values at this dosage was also lower in the isolate CKD (6.780 days) followed by EKM2 (7.095 days). The highest LT_{90} value was observed in the isolate M4 with 8.788 days. CKD has shown lowest time for the 50 per cent and 90 per cent mortality of the *Galleria* larvae followed by EKM2 at 1x 10^9 spores/ ml of dose.

The results are showing that both LT_{50} and LT_{90}values were decreased with increase in concentration of the spores/ml. At the highest dose of 1x 10^9 spores/ ml, isolate CKD has recorded lower LT_{50} and LT_{90}values. At all other doses EKM2 ha recorded the lower LT_{50} and LT_{90}values. The isolate M4 has recorded the highest LT_{50} and LT_{90}valuesat all the concentrations spores. Hence, EKM2 and CKD are proved to be elite and virulent isolates when applied at a higher concentration and M4 is found to be less virulent compared to the other isolates.

Table 25. Dose- mortality responses of larvae of *Galleria mellonella* with *Metarhizium anisopliae*

Dose (cfu/ml)	*M. anisopliae* isolates	LT_{50} (Days)	Fiducial Limit (95%)	Chi-square	Degrees of freedom	slope	LT_{90} (Days)	Fiducial Limit (95%)
1 x 10^5	**CKD**	8.806	8.173-9.778	7.630	8	6.478	13.887	12.146-17.275
	EKM2	7.989	7.592-8.437	2.157	8	9.825	10.788	9.943-12.281
	M4	9.460	8.781-10.644	4.285	8	7.397	14.097	12.074-19.067
1 x 10^6	**CKD**	7.278	6.972-7.653	5.536	8	10.017	9.771	9.103-10.857
	EKM2	7.125	6.767-7.480	5.237	8	10.385	9.466	8.859-10.427
	M4	7.507	7.099-7.945	5.283	8	8.660	10.553	9.691-12.032
1 x 10^7	**CKD**	6.388	6.037.6.732	5.421	8	9.450	8.729	8.228-9.443
	EKM2	6.621	6.187-7.024	9.700	8	11.876	8.488	7.906-9.427
	M4	7.114	6.751-7.478	5.116	8	10.033	9.547	8.912-10.558
1 x 10^8	**CKD**	5.912	5.487-6.313	8.814	8	9.624	8.033	7.415-9.055
	EKM2	5.414	5.099-5.719	6.221	8	9.714	7.335	6.854-8.050
	M4	6.390	6.009-6.763	5.523	8	8.310	9.115	8.443-10.158
1 x 10^9	**CKD**	5.099	4.480-5.626	18.322	8	10.359	6.780	6.097-8.188
	EKM2	5.359	5.055-5.653	1.450	7	10.518	7.095	6.641-7.782
	M4	6.124	5.745-6.489	7.350	8	8.171	8.788	8.147-9.767

4.10. Biochemical analysis of stress tolerance and virulence

The selected temperature tolerant isolate (EKM2) and drought tolerant isolate (CKD) were biochemically analysed and compared to the control of each isolates. The total protein content, trehalose content, catalase activity, peroxidase activity, protease activity, lipase activity and chitinase activity were analysed using standard protocols.

The variations in the biochemical activity of the selected tolerant isolates are depicted in Table 26 and 27.

4.10.1. Total protein content in stress-tolerant isolates of *Metarhizium anisopliae*

The total protein content of the selected thermo-tolerant isolates (EKM2) and its control were calculated (Table 26). The thermo-tolerant isolate (41^{o}C), EKM2 has recorded a total protein content of 0.354 mg/ml which is significantly higher when compared to the total protein content (0.215) of the isolate grown at room temperature (26 ± 2 ^{o}C) (Plate 32).

The total protein content of the drought-tolerant isolate (CKD) was recorded 0.373 mg/ml. The isolate CKD when grown at room temperature has shown a protein content of 0.259 mg/ml which is significantly low compared to the tolerant one (Table 27).

4.10.2. Trehalose content in stress-tolerant isolates of *Metarhizium anisopliae*

The trehalose content in the isolate EKM2 was estimated and results are depicted in Table 26 (Plate 33). The thermo-tolerant isolate EKM2 has shown a trehalose content of 1.954 mg/min/g tissue weight which was significantly higher when compared to the trehalose content of the same isolate grown at room temperature (1.074 mg/min/g tissue weight).

The trehalose content in the drought tolerant isolate EKM2 was recorded 1.970 mg/min/g tissue weight and was significantly superior compared to the control (1.224 mg/min/g tissue weight) (Table 27).

4.10.3. Activity of enzyme catalase in stress tolerant isolates of *Metarhizium anisopliae*

The catalase activity of the Thermotolerant isolate EKM2 and its control were calculated and the results are presented in the Table 26. The catalase activity ranged between 0.237 EU/min/mg proteins in the control to 0.386 EU/min/mg protein for the thermo-tolerant isolate. The catalase activity of the thermo-tolerant isolate CKD was significantly higher compared to the isolate when it is grown at room temperature.

The catalase activity of the drought tolerant isolate, CKD was recorded 0.384 EU/min/mg protein. The catalase activity of the same isolate when grown at

room temperature shown a reduced activity of 0.240 EU/min/mg protein. The results showed that the catalase activity of the drought tolerant CKD is significantly higher compared to the control (Table 27).

Table 26. Biochemical assay of thermo-tolerant isolate of *Metarhizium anisopliae*

Isolate	Temperature	Total protein (mg/ml)	Trehalose (mg/min/g tissue weight)	Catalase (EU/min/mg protein)	Peroxidase (EU/min/g tissue weight)	Protease (EU/min/mg protein)
EKM2 induced	41 ^{o}C	0.354^{a}	1.954^{a}	0.386^{a}	0.0230^{a}	0.490^{b}
EKM2 Control	Room temperature (26 ± 2 ^{o}C)	0.215^{b}	1.075^{b}	0.237^{b}	0.0120^{b}	0.467^{c}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

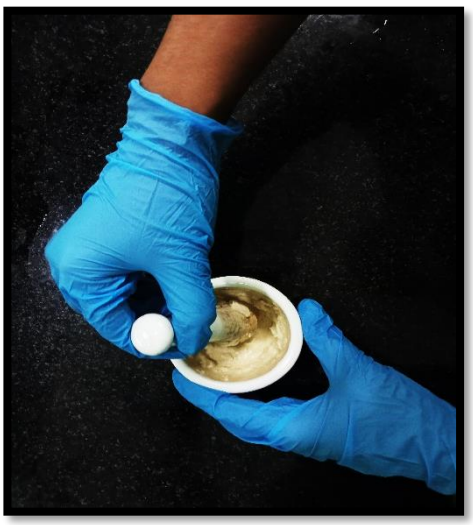
Table 27. Biochemical assay of drought tolerant isolate of *Metarhizium anisopliae*

Isolate	Drought	Total protein (mg/ml)	Trehalose (mg/min/g tissue weight)	Catalase (EU/min/ mg protein)	Peroxidase (EU/min/g tissue weight)	Protease (EU/min/mg protein)
CKD induced	40% PEG concentration	0.373^{a}	1.970^{a}	0.384^{a}	0.0236^{a}	0.621^{a}
CKD Control	Room temperature (26 ± 2 ^{o}C)	0.259^{b}	1.224^{b}	0.240^{b}	0.0130^{b}	0.483^{bc}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

4.10.4. Activity of enzyme peroxidase in stress tolerant isolates of *Metarhizium anisopliae*

The peroxidase activity of thermotolerant isolate EKM2 was estimated and compared with the peroxidase activity of its control (Table 26). The thermo-tolerant isolate has shown a peroxidase activity of 0.0230 EU/min/g tissue weight which was significantly higher compared to the peroxidase activity of the control (0.0120 EU/min/g tissue weight)

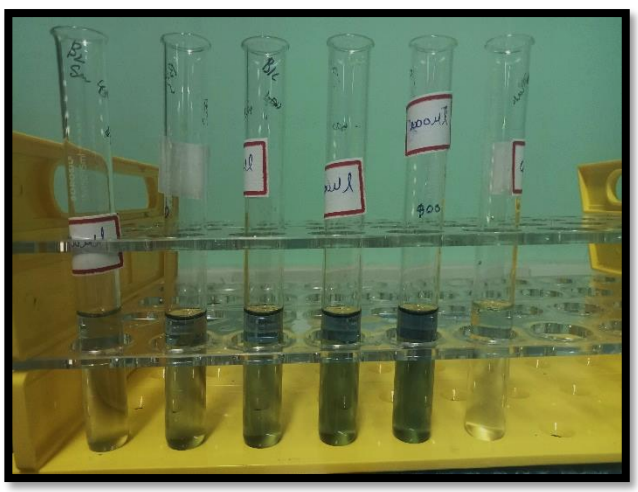


i) Grinding mycelial mat in sodium phosphate buffer

ii) sonicate for 8 minutes

iii) Centrifugation at 10000 rpm for 20 minutes

iv) Adding reagents



v) After incubation – intensity of blue colour indicates protein content

Plate 32. Estimation of total protein content

i) Adding reagents to the enzyme extract

iii) Kept in water bath at 90 ^{o}C

iv) After heating intensity of green colour denotes the trehalose content

Plate 33. Estimation of trehalose content

The peroxidase activity of drought tolerant isolate CKD was recorded as 0.0236 EU/min/g tissue weight. The peroxidase activity of the control grown at room temperature was recorded significantly lower activity (0.0130 EU/min/g tissue weight) compared to the tolerant one (Table 27)

4.10.5. Protease activity in stress tolerant isolates of *Metarhizium anisopliae*

The protease activity of thermo-tolerant isolate EKM2 was recorded higher (0.490 EU/min/mg protein) compared to the protease activity of the control (0.467 EU/min/mg protein). The thermo-tolerant isolate EKM2 showing higher protease activity compared to the control as depicted in Table 26 (Plate 34).

The protease activity of the drought-tolerant isolate CKD and its control which is grown at room temperature was estimated and compared (Table 27). The thermo-tolerant isolate has shown a protease activity of 0.621 EU/min/mg protein which was significantly higher compared to that of its control (0.483 EU/min/mg protein)(Plate 34).

4.10.6. Activity of enzyme lipase in stress tolerant isolates of *Metarhizium anisopliae*

The lipase activity of the stress tolerant isolates and their control were estimated using titrimetric method. The lipase activity was estimated at 5 min interval for 25 min (Plate 35). The results of the experiment are presented in Table 28 and 29.

The lipase activity of thermo-tolerant isolate (EKM2) and its control were estimated and the results are given in Table 28. The lipase activity of the thermo tolerant isolate was found to be significantly high compared to that of control at all-time intervals. After 5 min of the reaction, the thermo-tolerant EKM2 has shown an activity of 20.667 µmol fatty acid/ml which is significantly high compared to the control (7.000 µmol fatty acid/ml). At 10 min of the reaction the lipase activity of the tolerant isolate and the control was 14.333 µmol fatty acid/ml and 6.667 µmol fatty acid/ml respectively. At 15 min the thermo-tolerant isolate recorded 15.667 µmol fatty acid/ml of lipase activity which is significantly higher compared to the control (5.000 µmol fatty acid/ml). At 20 min of reaction both stress tolerant one and control showed 7.000 µmol fatty acid/ml of lipase activity. At 25 min of reaction again the thermo-tolerant isolate has shown a higher lipase activity (10.000 µmol fatty acid/ml) than

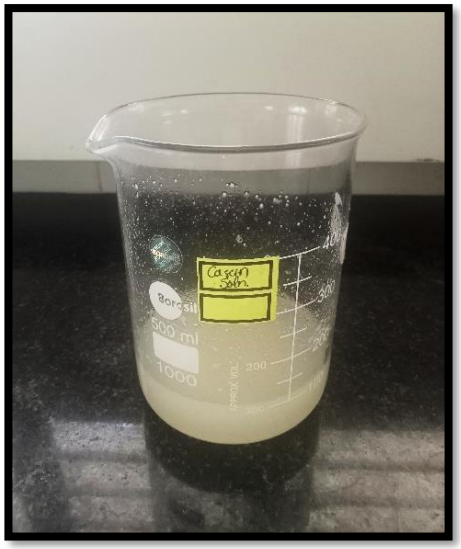
control (8.667 µmol fatty acid/ml). The activity of lipase was fluctuating with time and the highest activity of lipase in the thermo-tolerant EKM2 was found at 5min which was reduced at10 and 15 min. The activity was again reduced at 20 min but there was again an increase at 25 min of reaction**.**

Table 28. Lipase activity of thermo-tolerant isolates (EKM2) *of Metarhizium anisopliae*

Isolate	Lipase activity (µmol fatty acid/ml)					
	5 min	**10 min**	**15 min**	**20 min**	**25 min**	**Mean**
EKM2 Induced	20.667[a]	14.333[b]	15.667[b]	7.000[d]	10.000[cd]	13.533[a]
EKM2 Control	7.000[d]	6.667[d]	5.000[e]	7.000[d]	8.667[c]	6.867[b]

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

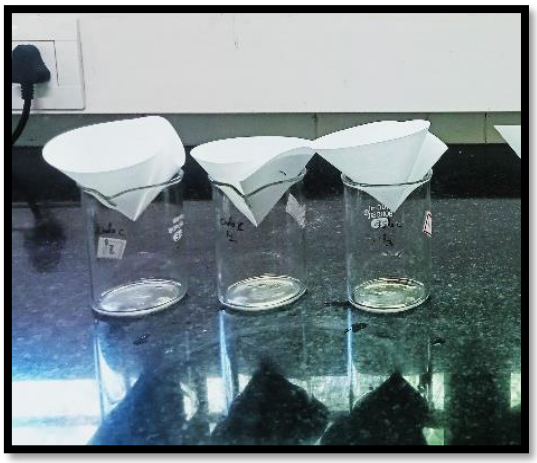
The lipase activity of drought tolerant isolate (CKD) and the control was also estimated and results are presented in Table 29. The lipase activity of the drought-tolerant isolate CKD was higher at all-time intervals compared to the control. At 5 min of the reaction, both the tolerant isolate and the control have shown a lipase activity of 5.000 µmol fatty acid/ml. At 10 min of reaction, the drought tolerant isolate and control has shown a lipase activity of 6.000 µmol fatty acid/ml and 7.667 µmol fatty acid/ml respectively. At 15 min of reaction the drought tolerant one has shown a lipase activity of 8.333 µmol fatty acid/ml which is significantly high compared to the control (5.000 µmol fatty acid/ml). At 20 min of reaction the stress tolerant isolate has shown 7.333 µmol fatty acid/ml of lipase activity and the control showed 6.000 µmol fatty acid/ml of lipase activity. At 25 min of the reaction the drought tolerant isolate has shown a lipase activity of 11.00 µmol fatty acid/ml which is significantly higher compared to that of its control (5.667 µmol fatty acid/ml). The highest lipase activity of the drought tolerant isolate was observed at 25 min of the reaction followed by at 15 min. The activity at 5 min was the lowest and increased after 5 min.



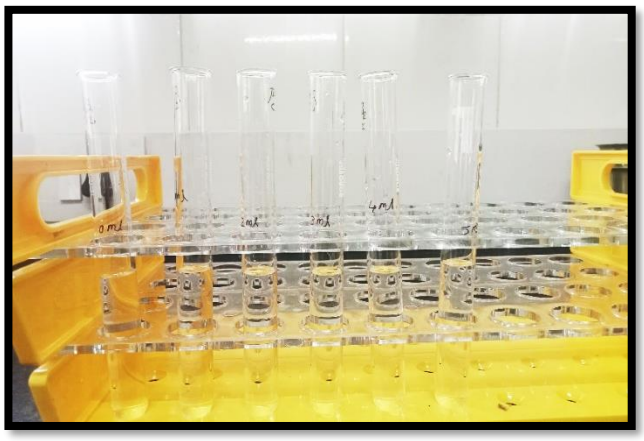
i) Preparation of casein solution solution

ii) Adding casein solution

iii)Incubation at 40 ºC



iv) Filtration of the contents using Whatman No. 1 filter paper



v) Adding reagents – Intensity of blue colour indicates protease activity

Plate 34. Estimation of Protease activity

i) Olive oil –gum Arabic solution added to the titration cocktail

ii) Titrating contents of each flask with 0.05 N NaOH until the appearance of blue colour

Plate 35. Estimation of lipase activity

Table 29. Lipase activity of drought-tolerant isolates (CKD) of *Metarhizium anisopliae*

Isolate	Lipase activity (µmol fatty acid/ml)					
	5 min	10 min	15 min	20 min	25 min	Mean
CKD Induced	5.000^{d}	6.000^{cd}	8.333^{b}	7.333^{bc}	11.00^{a}	7.533^{b}
CKD Control	5.000^{d}	7.667^{bc}	5.000^{d}	6.000^{cd}	5.667^{cd}	5.867^{c}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

4.10.7. Chitinase activity in stress tolerant isolates of *Metarhizium anisopliae*

The chitinase activity of the stress tolerant isolates and their control was estimated and compared with each other. The activity of chitinase was recorded for 5 days after incubation and the results are presented in Tables 30 and 31(Plate 36).

Table 30. Chitinase activity in thermo-tolerant isolate (EKM2) of *Metarhizium anisopliae*

Isolate	Chitinase activity (µg of N- acetyl glucosamine/min/ml of broth)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Mean
EKM2 Induced	1.607^{a}	0.205^{g}	0.635^{e}	0.898^{d}	1.140^{b}	0.917^{a}
EKM2 Control	1.140^{c}	0.096^{h}	0.102^{h}	0.077^{h}	0.548^{f}	0.393^{b}

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMR

The thermotolerant isolate EKM2 and its control were analysed for the chitinase activity for 5 days and the results are presented in Table 30. The activity of chitinase was higher during the initial days and reduced after that and increased again on 5th day for the thermo-tolerant isolate. On the first day, chitinase activity of EKM2 was 1.607 µg of N- acetyl glucose amine/min/ml of broth and that of control was 1.140 µg of N- acetyl glucose amine/min/ml of broth. The activity of chitinase for thermo-tolerant EKM2 on the second day was 0.205 µg of N- acetyl glucose amine/min/ml of broth and 0.0960 µg of N- acetyl glucose amine/min/ml of broth for the control. On the 3rd day it was again increased to 0.635 µg of N- acetyl glucose

amine/min/ml of broth for the thermo-tolerant isolate and 0.102 µg of N- acetyl glucose amine/min/ml of broth for the control. In the 4th day, again showed hype in the chitinaseactivity for the thermo-tolerant EKM2 with 0.898 µg of N- acetyl glucose amine/min/ml of broth. But the control showed a decrease in the chitinase activity in the 4th day (0.077 µg of N- acetyl glucose amine/min/ml of broth). On the 5th day the thermo-tolerant EKM2 has shown a higher chitinase activity (1.140 µg of N- acetyl glucose amine/min/ml of broth) followed by the control with 0.548 µg of N- acetyl glucose amine/min/ml of broth. The results reveal that the thermo-tolerant isolate EKM2 has showed a higher chitinase activity compared to the chitinase activity of the control.

Table 31. Chitinase activity in drought tolerant isolate (CKD) of *Metarhizium anisopliae*

Isolate	Chitinase activity (µg of N- acetyl glucose amine/min/ml of broth)					
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**
CKD Induced	0.978[d]	1.373[c]	1.369[c]	1.575[b]	3.062[a]	1.672[a]
CKD Control	0.108[e]	0.061[e]	0.061[e]	0.073[e]	0.069[e]	0.074[e]

*****Mean of three observations; figure followed by same letter do not differ significantly according to DMRT

The chitinase activity of the drought-tolerant isolate CKD and its control were analysed for 5 days and the results are depicted in table 31. The chitinase activity of the drought tolerant isolate has shown an increasing trend while the control has shown a fluctuating chitinase activity. The chitinase activity of drought tolerant CKD and its control on the first day were 0.978 µg of N- acetyl glucose amine/min/ml of broth and 0.108 µg of N- acetyl glucose amine/min/ml of broth respectively. The drought tolerant CKD has shown a rise in the chitinase activity (1.373 µg of N- acetyl glucose amine/min/ml of broth) on the 2nd day while the chitinase activity of the control was decreased to 0.061 µg of N- acetyl glucose amine/min/ml of broth in the control. The chitinase activity on the 3rdday of incubation was 1.369 µg of N- acetyl glucose amine/min/ml of broth for the drought tolerant one and 0.061 for the control. On day four, the drought tolerant CKD has shown an increase in the chitinase activity (1.575 µg of N- acetyl glucose amine/min/ml of broth). The control has also shown a

40 g chitin was dissolved in 250 ml ice cold HCL

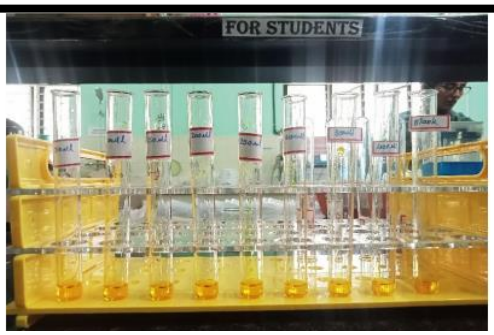
Glass wool was used to filter the mixture

Collected into 2l ice cold water under stirring

The filtrate with a gelatinous white material was separated using whatman no. 1 filter paper

Obtained colloidal chitin was then repeatedly washed with tap water until it gains neutral pH

M. anisopliae was cultured in 250 ml flasks with 15 ml of sterile water containing 1.5% colloidal



1 ml of the supernatant was taken into test tube and 1 ml of DNS reagent was added

Tubes were heated at 95^{o}C for 10 minutes and absorbance was measured at 540 nm

Plate 36. Estimation of chitinase content

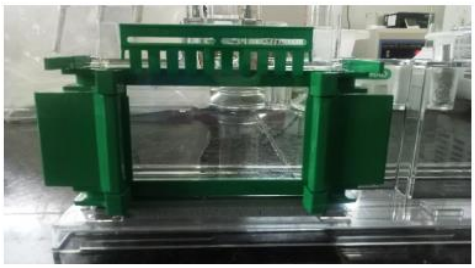
chitinase activity of 0.073 µg of N- acetyl glucose amine/min/ml of broth on the 4th day. The highest chitinase activity of the drought-tolerant CKD was reported on the 5th day (3.062 µg of N- acetyl glucose amine/min/ml of broth). The chitinase activity of the control on the 5th day was 0.074 µg of N- acetyl glucose amine/min/ml of broth. From the results it is evident that the chitinase activity of the drought-tolerant CKD was higher on all days compared to the control.

4.10.8. Protein profiling using SDS-PAGE

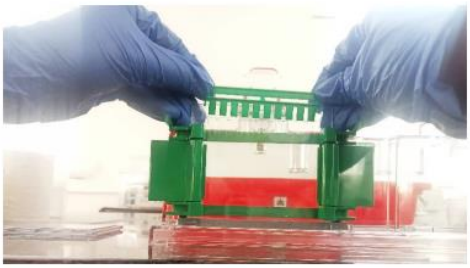
The protein profilingof selected tolerant isolates of *Metarhizium anisoplaie* were done using SDS-PAGE (Plate 37). The thermo tolerant isolate EKM2 and drought tolerant isolate CKD and their controls were subjected to SDS PAGE. The protein profile of the isolates were obtained after SDS PAGE was compared with prestained protein ladder with 12 bands (11 kDa to 180 kDa), which was loaded as marker. The thermo tolerant EKM2 had expressed number of bands which was absent in its control. The isolate had over expressed the protein at a molecular weight of 25 kDa and 35 kDa. The bands were thicker at this range when compared to the control. Two bands of molecular weight 100 kDa and 180 kDa were found extra in the thermo tolerant isolate EKM2 when compared to the control. At the same time the protein at a range of molecular weight 75 kDa was absent in thermo tolerant EKM2 and present in the control. So it was concluded that this protein was down regulated in EKM2 (Plate 37).

The protein profiling of drought tolerant isolate CKD and its control were also done using SDS PAGE. The protein profiles of both control and drought tolerant CKD were similar and there was no extra bands or thicker bands in the isolate CKD compared to the control. So it is concluded that the drought induction does not up regulated any proteins in the isolates CKD.

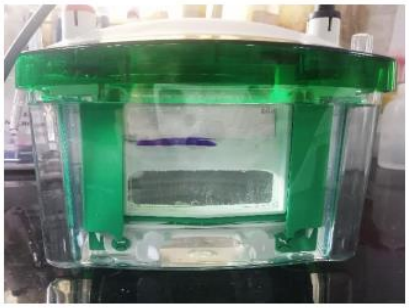
Hence, it is concluded that the temperature induction had up regulated heat shock proteins of range 25 kDa to 35 kDa and expressed heat shock proteins 100 kDa and 180 kDa molecular weight in response in the isolate EKM2. This is confirming the thermo tolerance of the isolate EKM2. The drought induction on the other hand did not showed the presence of any heat shock protein in CKD when compared to the control. Both CKD and control had shown similar protein profiles and proved that drought did not cause expression of heat shock proteins.



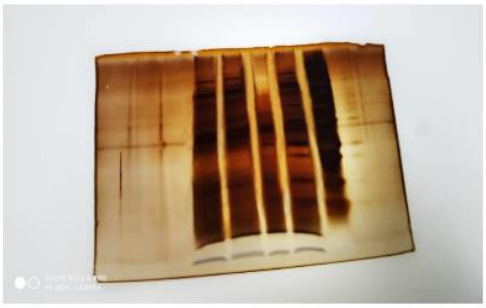
Gel casting



Removal of combs to create wells



Running the samples in SDS PAGE unit for 45 minutes



Silver staining of the gel

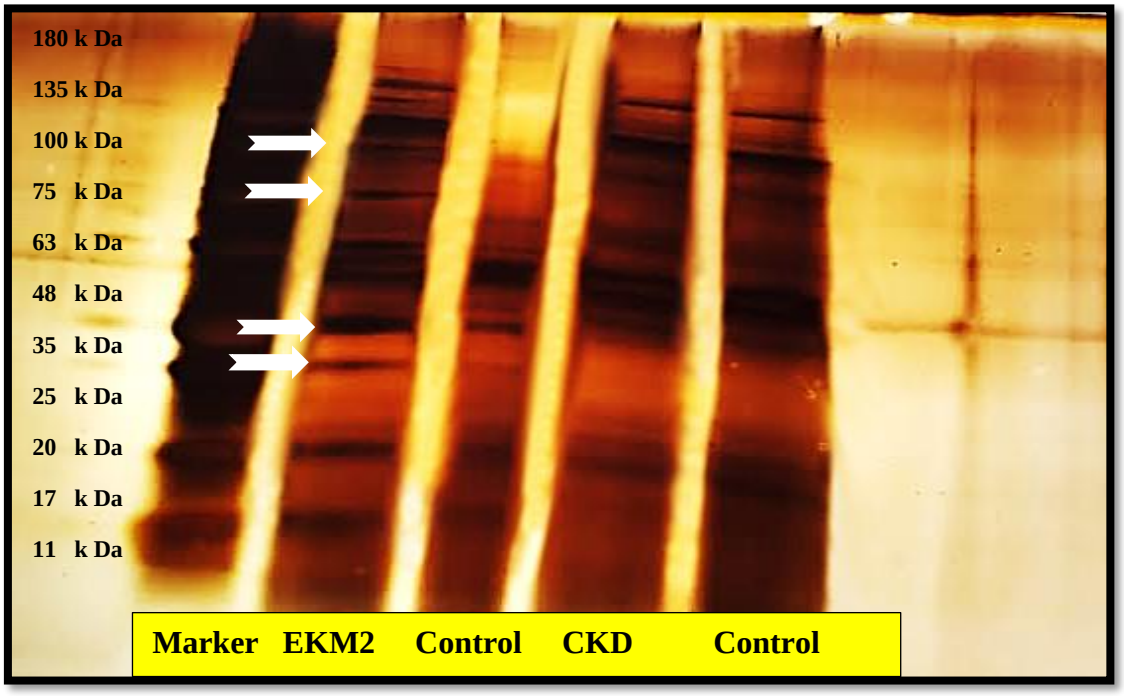


Plate 37. Estimation of heat shock protein content using SDS PAGE

DISCUSSION

5. DISCUSSION

The anamorphic stages of *Metarhizium anisopliae* have a broad insect host range. The development of pest resistance to several chemical pesticides and public concern about the negative effects of wide spread use of chemicals on human health, food safety, and the environment have sparked interest in the use of biologically based pest management methods (Moore and Prior, 1993). The ascomycetous fungi, *Metarhizium anisoplie* Sorokin. is found all over the world, is recognized as a biological pest control agent (Goettel *et al.*, 2005). The species in this genus live in the soil as saprobes, endophytes, and dwellers of the rhizosphere. They also exhibit intricate symbioses as insect pathogens and antagonistic relationships with fungi that cause fungal plant infections (Vega *et. al*., 2009).

The vulnerability of this fungus to numerous abiotic stimuli, such as the need for high relative humidity, sensitivity to UV radiation, and high temperatures (>32 °C), frequently prevents effective application of the fungus (Fernandes *et al*., 2015). The need to develop strains of *Metarhizium* sp. with enhanced tolerance to abiotic stress conditions and increased biocontrol efficiency in the field has thus become a necessary. The discussions on the experiments are furnished in the chapter.

5.1. Cultural and morphological characterization of *Metarhizium anisopliae* isolates

The cultural, morphological and molecular characterization of the *M. anisopliae* isolates CKD, EKM2 and M4 were carried out. The isolate CKD was white in colour initially and gradually changed to dark green in colour with circular wavy markings and dark green conidia. The isolate EKM2 was yellowish-green in colour initially and changed to a dirty white in colour later becomes pale yellowish green, and finally becomes dark yellowish green with dark green spores. Isolate M4 was white in colour that changed to yellow and then to light green to olive colour. This is in conformity with the findings of Bischof *et al*., (2009) in which they reported that, the different isolates of *M. anisopliae* complex had white colonies that typically turn yellow when the conidia begin to mature. Bai *et al*., (2015) had also found that, *Metarhizium* isolate colonies had greenish colour and round shape. Ayala *et al*., (2017) also reported that, the preliminary characterization showed that all the

Metarhizium isolates screened produced typical greenish conidial masses on the culture plates with smoth plate reverses.

Conidia of the isolates CKD and EKM2 were formed as clusters at the tip of phialides and the hyphae are branched with short phialides with thick and multiple-branched clusters of conidia. But in case of M4, conidia are formed as both clusters and chains from the tip of phialide. Maximum phialide size was observed for the isolate CKD (13.14x4.72µm) and it was immediately followed by EKM2 (9.33x3.18µm), while M4 recorded smallest phialide size of 6.82x2.02µm (Fig. 1). Studies on spore characters have shown that spore size was largest for EKM2 (8.33x2.41µm) and was followed by CKD (7.71x1.4) and the least spore size was observed for M4 (6.97x1.34 µm). Mycelial thickness was also more in EKM2 (7.29 µm) followed by CKD (6.47 µm) and M4 with thinner mycelia (5.12 µm) (Fig. 2). Results are in conformity with the findings of Bischof *et al*., (2009) who found that, the mean length and width of phialides of *M. anisopliae*are 6.82 µm and 2.02 µm, respectively with a mycelial thickness of 5.12 µm. Sorokin (1883) found that the genus *Metarhizium* gave the spore dimensions of 4.0 X 1.6 µm.

Moslim and Kamarudin (2014) had studied spore characters of EPF, through microscopic observations and reported that *Beauveria* isolates were with the globose to subglobose conidia with hyaline hyphae whereas *Metarhizium* isolates were characterized with ellipsoid, and cylindrical spore shapes.

5.2. Molecular characterization of *Metarhizium anisopliae* isolates

The nucleotide sequence of CKD captured from NCBI showed more than 99 per cent similarity with 99 per cent query coverage to *M. anisopliae* isolates, HN26Z01, SC36A02 and isolate SC50C05. The nucleotide sequence of EKM2 received from NCBI revealed 99. 58 per cent similarity with 100 per cent query cover to the *M. anisopliae* isolates MaGD46, HA02B01, HE15A01, HE19B02 and HE05B02. The nucleotide sequence of M4 obtained from NCBI showed 99.79 per cent identity with 96 per cent query coverage to *M. anisopliae* isolates BUM1900, M9, IIHR isolate and M-63. This is in conformity with the study of Garcia *et al*., (2018) who reported that, amplification and sequencing of the ITS-rDNA region of entomopathogenic fungi have considerably facilitated the detection of fungal isolates particularly *B. bassiana* and *M. anisopliae*.

5.3. Screening of *Metarhizium anisopliae* isolates for temperature tolerance

Temperature is one of the most important parameter for the growth and establishment of entomopathogens. The ability to grow, sporulate and effectively control the pests in different abiotic stresses in the field makes the entomopathogenic fungi successful. The isolates CKD, EKM2 and M4 were screened at temperatures of 30, 32, 34, 36, 38 and 40^{o}C in order to select the thermo-tolerant isolate.

5.3.1 Screening of *Metarhizium anisopliae* isolates for temperature tolerance on potato dextrose broth (PDB)

At most of the temperature levels, isolate EKM2 has shown highest mycelial weight and M4 has shown the least mycelial weight. The isolate CKD has shown intermediate mycelial weights at all temperatures. At 30^{o}C, the isolate CKD showed the highest mycelium weight (5.122g) followed by EKM2 (4.221g) and the least mycelial weight was observed in the isolate M4 (0.606). The highest mycelial weight at a temperature of 38^{o}C was observed in the isolate EKM 2 (6.221g) followed by CKD (0.897g) and the lowest biomass was observed in the isolate M4 (0.328g). None of the isolates survived at 40^{o}C. The sporulation pattern has also been observed and the isolates CKD and EKM2 has shown higher sporulation at all the temperature levels while the sporulation of M4 was very low at all levels of temperature.Among the three isolates, EKM2 has recorded highest mycelial weight and moderate sporulation and was found to be temperature tolerant and it was followed by CKD with better mycelial weight and highest sporulation. The isolate M4 with low mycelial weight and least sporulation at all the levels of temperature didn't grow from 38^{o}C onwards and hence considered as a susceptible isolate to temperature.This is in tune with the findings of Dimbi *et al*., (2004) who studied the growth and conidial germination of six *M. anisopliae* isolates and their virulence against African tephritid fruit flies and concluded that the optimum temperature for their growth and conidial germination was 25°C. As per Gillespie and Jone (2002), at 25 and 30°C, 95 per cent of *M. anisopliae* conidia germinated in 10-14 h compared with 14-25 h for *B. bassiana* strains. Ying and Feng (2006), also reported that, conidial tolerances of the *M. anisopliae* isolates to the thermal stress of 48^{o}C were well represented by their LT_{50} (10–150 min), which were spanned much more widely than those from the isolates of *Beauveria bassiana* (10.1–61.9 min) under the same thermal stress. The

optimum temperature range for maximum conidial germination, high mycelial growth and high conidial production was respectively wide, 20 -33ºC and 13-29ºC for *M. anisopliae* and *B. bassiana* isolates studied (Membang *et. al*, 2021).

5.3.2 Screening of *Metarhizium anisopliae* isolates for temperature tolerance on potato dextrose agar (PDA)

Radial growth of the isolates at varied temperatures were measured to know the growth inhibition if any at higher temperatures.

At 30ºC, the lowest level of inhibition percentage was shown by CKD (52.77%) and highest level of inhibition was shown by the isolate M4 (69.62%). The isolate, EKM2 has also shown an inhibition of 55.55 per cent. At the highest temperature of 38ºC, highest inhibition was shown by EKM2 and M4 which were on par with each other (77.030 and 77.770 % respectively) and the lowest was shown by the isolate CKD (72.220). None of the isolates had shown growth at 40ºC. At all the temperature levels highest percentage inhibition was recorded by the isolate M4 and EKM2 and CKD recorded comparatively lesserpercentage inhibition than M4. This implies that EKM2 is superior to other isolates and M4 is least tolerant to thermal stress (Fig. 3). The isolate M4 showed very low sporulation at all temperature levels. At 30ºC CKD has showed highest sporulation followed by EKM2 and least sporulation was observed in M4. The sporulation was observed to be gradually reducing with increasing temperature levels. At the highest temperature of 38ºC, sporulation was highest in CKD followed by a moderate sporulation in EKM2 and least in M4. This is in concurrence with the study of Ekesi *et al*., (1999) and Maniania *et al.,* (2008) who found that entomopathogenic fungi in general, have the optimum radial growth at temperatures between 25°C and 30°C However, different strains vary in their tolerance to higher temperatures. Ouedraogo *et al.,* (1997) has also observed that strains of *M. anisopliae* are better adapted to higher temperatures (35°C) than strains of *M. flavoviride*. Thomas and Jenkins (1997) has also studied the effect of temperature on growth of *M. flavoviride* isolates and found different temperature optima for radial growth and conidial concentration.

Fig.1 Phialide characters of *Metarhiziumanisopliae*isolates

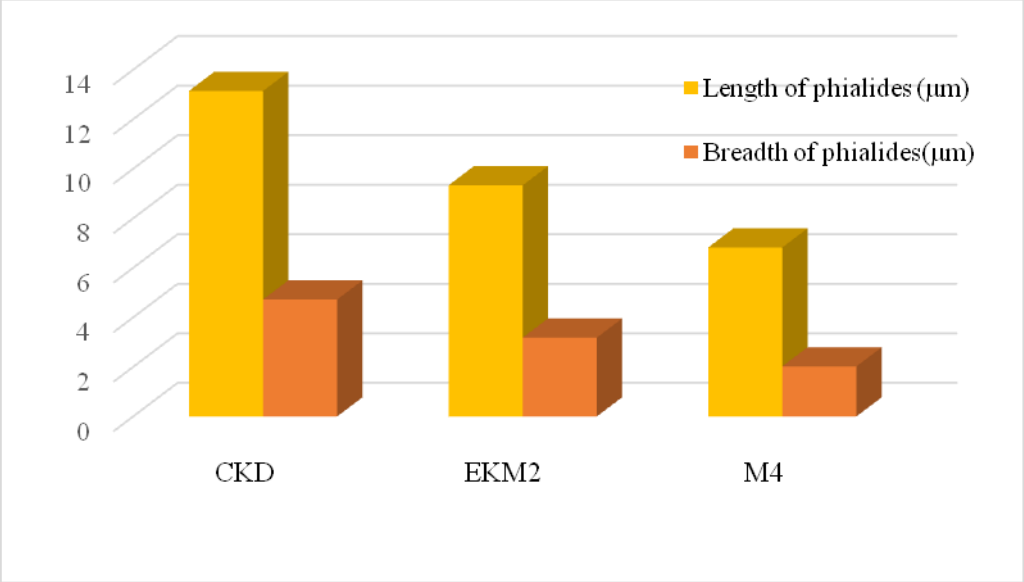
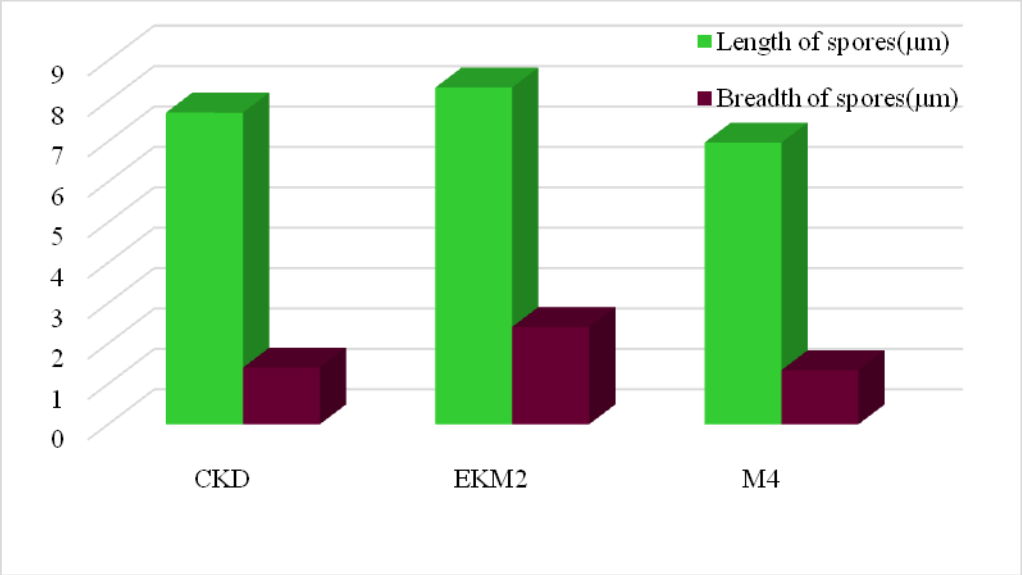


Fig. 2. Spore characteristics *of Metarhizium anisopliae* isolates



5.4. Screening of *Metarhizium anisolpiae* isolates for drought tolerance

Persistence of fungi at extreme moisture stress in the field is very important for successful biocontrol. The isolates with enhanced tolerance to moisture stress will be an added advantage for their efficacy in the field. The isolates were screened for drought tolerance at different polyethylene glycol (PEG) concentrations *viz*., 10, 20, 25, 27.5, 30, 32.5, 34, 36, 38, 39 and 40 per cent.

The isolate EKM2 displayed the highest mycelial weight (3.005g) at the lowest PEG content, 10%, followed by CKD (2.434g). The isolate M4, had the least growth (1.10g) when compared to the other isolates. Highest PEG concentration at which growth was noted was 39 per cent. None of the isolates have demonstrated growth at 40% PEG. At the highest PEG concentration, CKD has exhibited better biomass (2.172g), followed by EKM2 (2.055), and M4 has the lowest biomass detected (1.336). Therefore, CKD is proved to be superior to EKM2 and M4.The isolate, M4 have showed least weight and indicated drought susceptibility (Fig 4).The isolate CKD has exhibited the highest sporulation at all levels of PEG concentration, and was followed by EKM 2 with moderate sporulation. At all PEG concentration levels, M4 had the least amount of sporulation. This is in conformity with the study of Shah *et al*. (2005) who found increased virulence of conidia produced under osmotic stress (-75 MPa). Devi *et al.,* (2005) found that reduced water activity of -0.45 MPa did not affected the growth in majority of the *B. bassiana* isolates. Ibrahim *et al*., (2002) and Shah *et al.,* (2005) found that *M. anisopliae* produced on water-poor media had increased virulence to insects.

According to the results of the experiment, isolate CKD has higher biomass and more sporulation at the highest PEG concentration. Therefore, it was proved that CKD was drought tolerant, whereas isolate M4 was shown to be vulnerable since it had the lowest mycelial weight and least amount of sporulation. The isolate, EKM2 with moderate sporulation and was moderately tolerant. This is in consonance with the findings of Leland *et al.,* (2005) who found that there is an increase in spore output of *B. bassiana* (1.9 fold) and *M. anisopliae* (1.7 fold) at a lower concentration range (1-6%) of PEG (Polyethylene glycol) and also a 1.6 times spore yield enhancement *of M. anisopliae var. acridum* at a higher concentration range (5-30%) of the PEG.

Enhanced sporulation of *B. bassiana*, *B. brongniartii* and *M. anisopliae*induced by Polyethylene Glycol was reported in the study conducted by Humphreys *et al.*, (1989) and Knudsen *et al.*, (1991).

5.5. Inducing temperature tolerance in *Metarhizium anisopliae* isolates

The need for improving the *M. anisopliae* isolates for temperature tolerance is a necessity at the present scenario of increasing temperature. The improved thermotolerant isolates will have more persistence and hence better performance in the field conditions. The isolates CKD, EKM2 and M4 were subjected to continuous heat stress in an increasing manner to develop isolates with higher temperature tolerance.

5.5.1. Effect of induced temperature on mycelia weight of *Metarhizium anisopliae* isolates

The effect of induced temperature on mycelial weight of *Metarhizium anisopliae* isolates were studied in this experiment (Fig 6). All the three isolates were able to withstand a temperature of 41^{o}C, and none of them were able to grow at a temperature of 42^{o}C. With a modest amount of sporulation, the experiment clearly showed that EKM2 has the maximum weight of mycelia at all temperature settings, ranging from 1.36 g to 12.75 g. The isolate CKD followed EKM2 in mycelial weight, which ranged from 1.15g to 6.38g. The least sporulation and lowest mycelial weight (0.63 g -4.46 g) were recorded by M4 across all treatments. Thus, it is established that EKM2 is superior to other isolates in terms of thermal stress tolerance. The isolate M4 has been identified as the susceptible isolate, and the isolate CKD has been found to be only moderately tolerant to temperature stress. However, temperature induction has enhanced the performance of M4. This is in agreement with the study of James *et al.*, (1998) who found that, that *B. bassiana* germinated and grew most rapidly at a continuous temperature of 25-32°C. Thus with increase in temperature the weight of mycelia was found to be reduced for all the three isolates. However, when compared to the screening experiment where the isolates tolerated up to 39°C, the inducing technique have enhanced the tolerance upto 41°C. The tolerance was increased by 2°C with a higher mycelial weight.

Fig 3. Effect of temperature on the mycelial weight of *Metarhizium anisopliae* isolates

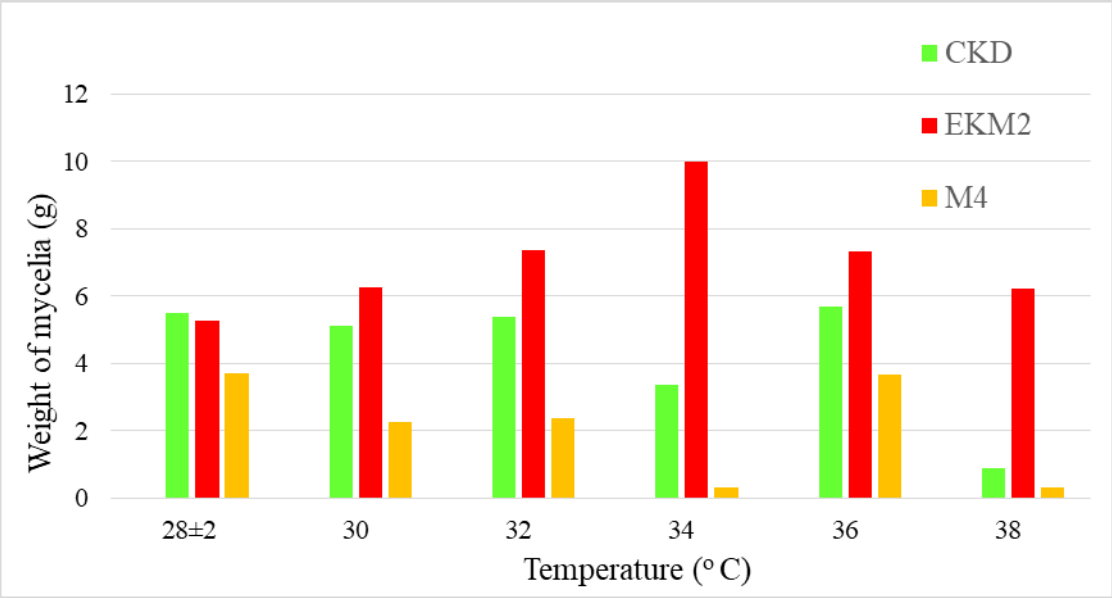


Fig 4. Effect of temperature on percentage inhibition of *Metarhizium anisopliae* isolates

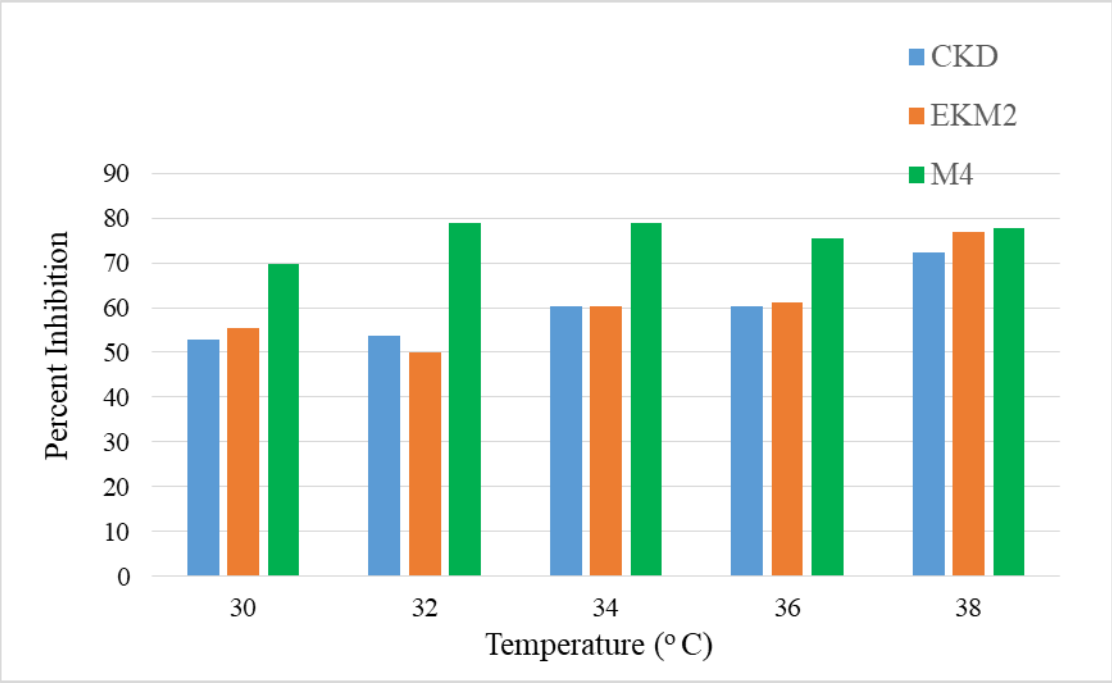
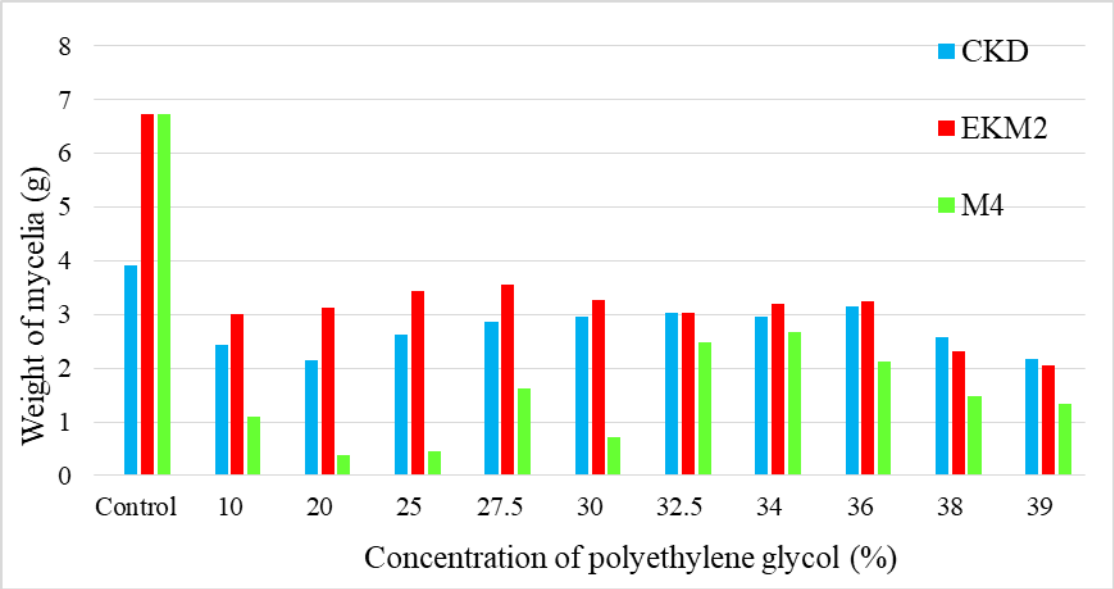


Fig 5. Effect of drought on mycelial weight of *Metarhizium anisoplaie* isolates



Li and feng (2009) reported that, conidial tolerance to 38-48ºC or colony growth rates at 10–35ºC differed greatly among *M. anisopliae* isolates. Zeng *et al.,* (2011) has also reported that compared to the wild type, the induced transformants showed a two-fold greater tolerance to UV radiation, a 1.3-fold greater tolerance to thermal stress (35ºC) and a 3-fold greater tolerance to low water activity (aw = 97.1%). Horaczek and Viernstein (2004) have also mentioned that, even after heat-resistant testin which the isolates were exposed to 45°C for 15 min, *M. anisopliae*did not lose viability. However, it was found that *B. bassiana*exposed for 15 min at 40 and 45°C, resulted in a 17 and 43 per cent reduction in activity, respectively.

5.5.2. Effect of induced temperature on growth inhibition of *Metarhizium anisopliae* isolates

At 35°C all the isolates recorded less than 20 per cent growth inhibition and the isolates M4 and CKD recorded lowest growth inhibition. The isolates M4 and CKD both demonstrated the lowest per centage inhibition at 35°C (11.11 and 18.5%, respectively) and isolate EKM2 showed the highest per centage inhibition (19.444 %). EKM 2 demonstrated the highest per centage inhibition at 40ºC. The isolate CKD has shown 55.553 per cent and M4 has shown 64.810 per cent, growth inhibition both had a low % inhibition compared to EKM2. EKM2 and M4 have demonstrated growth at the greatest temperature of 40º C. None of the isolate has grown at 42ºC. The isolate CKD sporulated more frequently at all temperatures, whereas the isolate EKM2 sporulated only moderately. At all of the treatments, the isolate M4 shown the least amount of sporulation. Schemmer *et al.,* (2016), also reported that *B. bassiana* and *M.anisopliae* which screened for radial growth ranged between 5.17 to 9.83 mm with conidial production of 9.08 - 31.87 X 10^5 conidia/mm with significant variability among isolates. This is in conformity with the study of Ouedraogo *et al.,* (1997) and Milner *et al.*,(2003), who showed that, the maximum upper temperature for mycelial growth in the *M. anisopliae* isolates used in this study was 36°C, which is consistent with other published results. Rangel *et al.,* (2008) also found that, that the mycelia of the two *M. acridum* isolates when developed for the first 24 h at 28°C and then incubated at 42°C for 10 d, survived and recommenced growth within two to three days after being transferred back to 28°C (Fig 7).

5.5.3. Effect of induced temperature on spore germination of *Metarhizium anisopliae* isolates

The isolate EKM2 had the maximum spore germination at 35°C (230.667 CFU/ml), followed by CKD (201.667 CFU/ml) and M4 was found to have the fewest colonies (40 CFU/ml). The number of colonies formed per ml substantially decreased after 40^{o}C. The isolate EKM2 had the most colonies (49 CFU/ml), followed by CKD (39 CFU/ml), and M4 had the fewest colonies (15 CFU/ml) at 40^{o}C. Only a smaller number of colonies from all the isolates are generated at 41^{o}C. EKM2 produced only 18 CFU/ml, followed by CKD, which also produced 11 CFU/ml, while M4 produced the fewest colonies per ml (3 CFU/ml).The findings indicate that EKM2 formed more colonies per ml at all temperature settings, followed by CKD. The isolate M4 had the fewest colonies per ml. The isolate EKM2 was shown to have the maximum spore germination at all temperature levels, proving it to be a thermotolerant isolate. Although the performance of CKD is bit lesser than EKM2, it was also exhibiting increased spore germination. The sensitive isolate was determined to be M4, which had the lowest spore germination at all temperatures (Fig. 8).This is in conformity with the findings of Belay *et al.*, (2017), who reported variationin the conidial variability of *B. bassiana*isolates (76.33 – 95.75%). Similar results were also obtained for Mkiga *et al.*, (2020), where they found 89.3 to 99 per cent variation in the conidial viability of 22 isolates of *B. bassiana* and *M. anisopliae.*Theconidial viability ranged from 85.3 to 99 per cent for 10 *Beauveria* and *Metarhizium* isolates was also reported by Habtegebriel *et al.,* (2016). The mean percentage spore germination of 26 (40%) isolates of *Beauveria* and *Metarhizium* was ranged from 85.43 to 99.67 per cent within 24 hrs (Bischof *et al.,* 2009). Gillespie and Jones (2002) also reported that, at 25 and 30°C, of *M. anisopliae* conidia germinated in 10-14 h compared with 14-25 h for *B. bassiana* strains. Onsongo *et al.,* (2003) had found that, the optimal temperature for conidial germination was observed to be between 25^{o}C and 30^{o}C for different *Metarhizium* isolates.

Fig 6. Effect of induced temperature on mycelial weight of *Metarhizium anisopliae* isolates

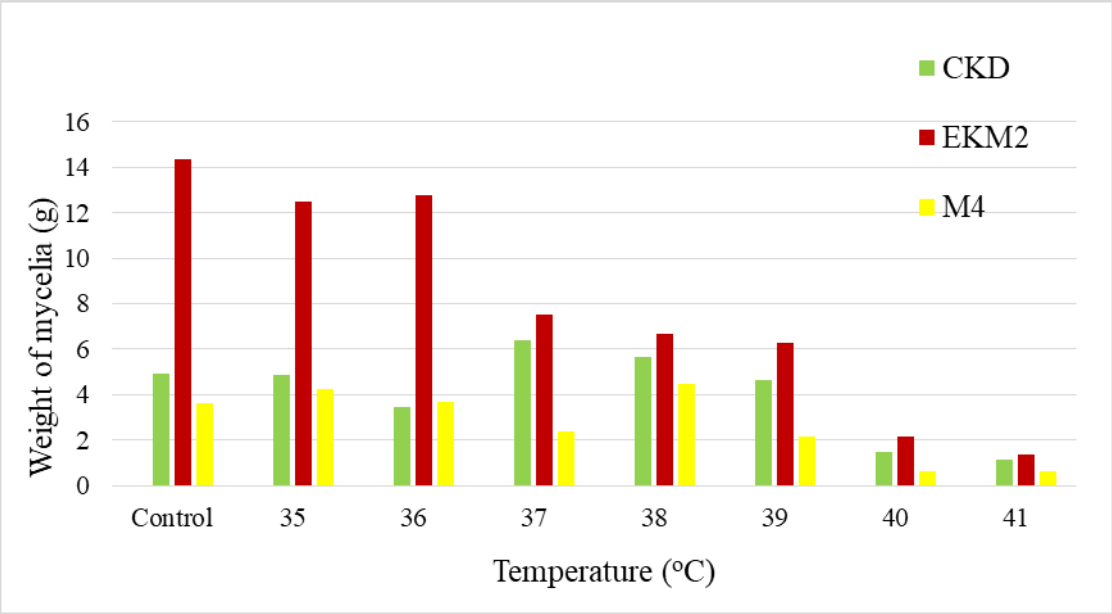
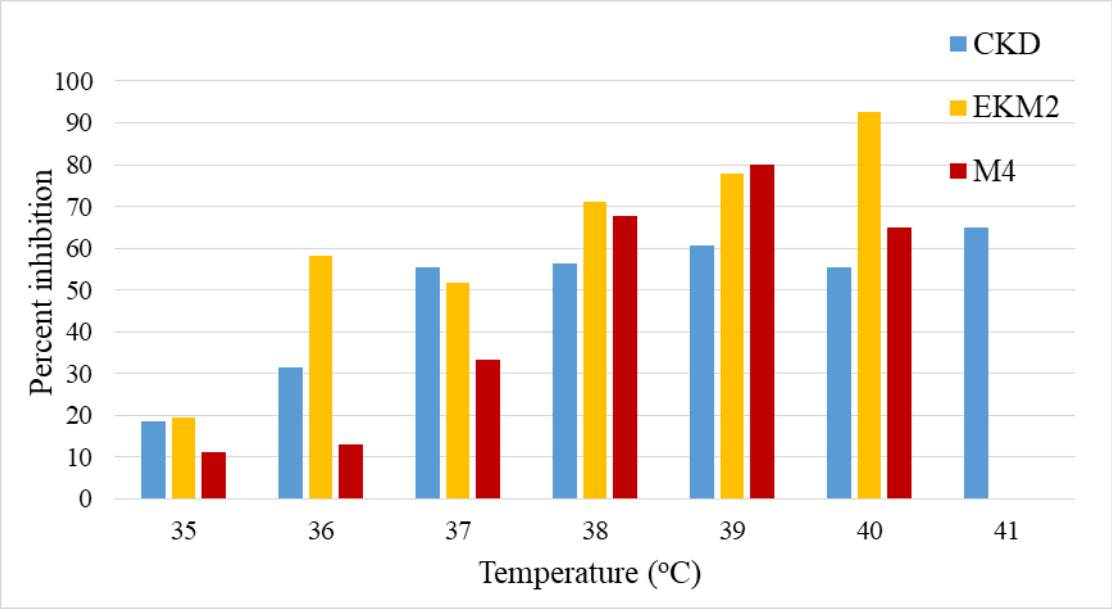


Fig 7. Effect of induced temperature on inhibition of *Metarhizium anisopliae* isolates



5.5.4. Effect of induced temperature on spore count of *Metarhizium anisopliae* isolates

The isolates, EKM2 has the largest spore count at all temperature levels, as per hemocytometer spore counts. The isolate EKM2 had the greatest spore count at 35°C (5.4 X 10^7/ml), followed by CKD (4.2 X 10^7/ml) and with 0.633 X 10^7 spores/ml, the isolate M4 had the lowest spore count. When the temperature was elevated to 40°C and 41 °C, the isolate EKM2 has recorded a higher spore count of 0.733X 10^7/ml and 0.783X 10^7/ml respectively proving its thermotolerance. It was followed by the isolate CKD (0.55 X 10^7/ml) and M4 recorded the least spore count (0.217 X 10^7/ml). The findings indicate that EKM 2 recorded the largest amount of spores per ml of PD broth at all temperature levels. The isolate M4 had the fewest spores overall at all temperature ranges. The results proved that the thermotolerance of EKM 2 which produced the largest amount of spores per ml of PD broth at all temperature levels. The isolate M4 had the fewest spores overall at all temperature ranges. Therefore, isolate M4 has been determined to be susceptible, isolate EKM2 to be thermotolerant, and isolate CKD to be moderately tolerant (Fig 9).

This is in agreement with the findings of Tefera and Pringle (2010), Arthurs and Thomas (2001) and Borisade and Magan (2014) who showed that, the optimum sporulation temperature was found to be 25ºC with a significant reduction of conidia at 30ºC for isolates of *M. anisopliae* and *B. bassiana.*The study by Arthurs and Thomas (2001) alsofound that the requirement for both appropriate temperatures (20–30°C) and high ambient RH (96 %) for sporulation of *M. anisopliae var. acridum*.

Fig 8. Effect of induced temperature on spore germination of *Metarhizium anisopliae* isolates

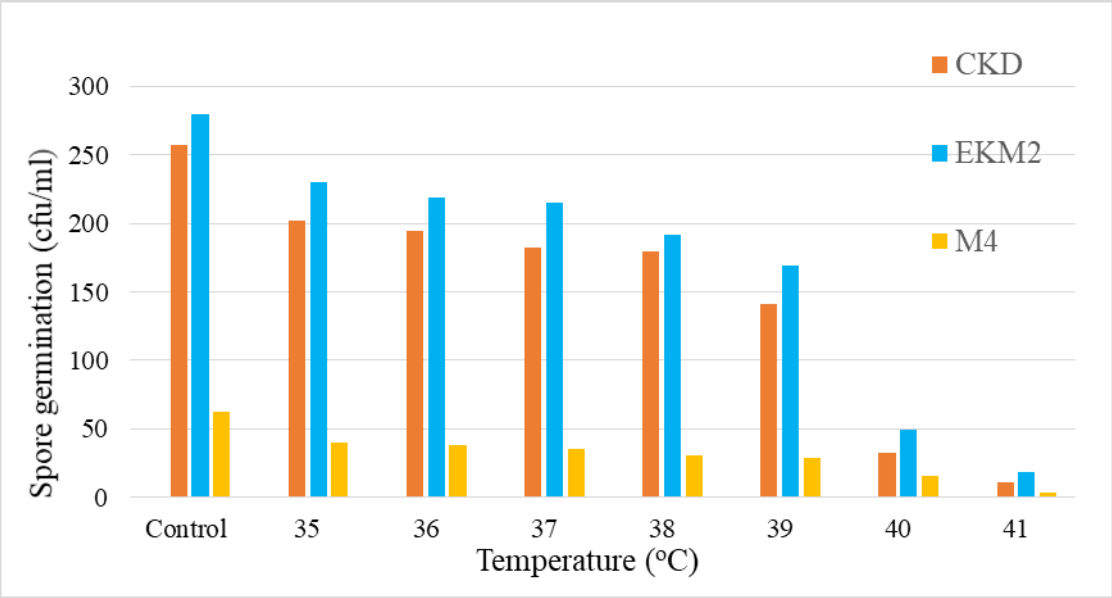
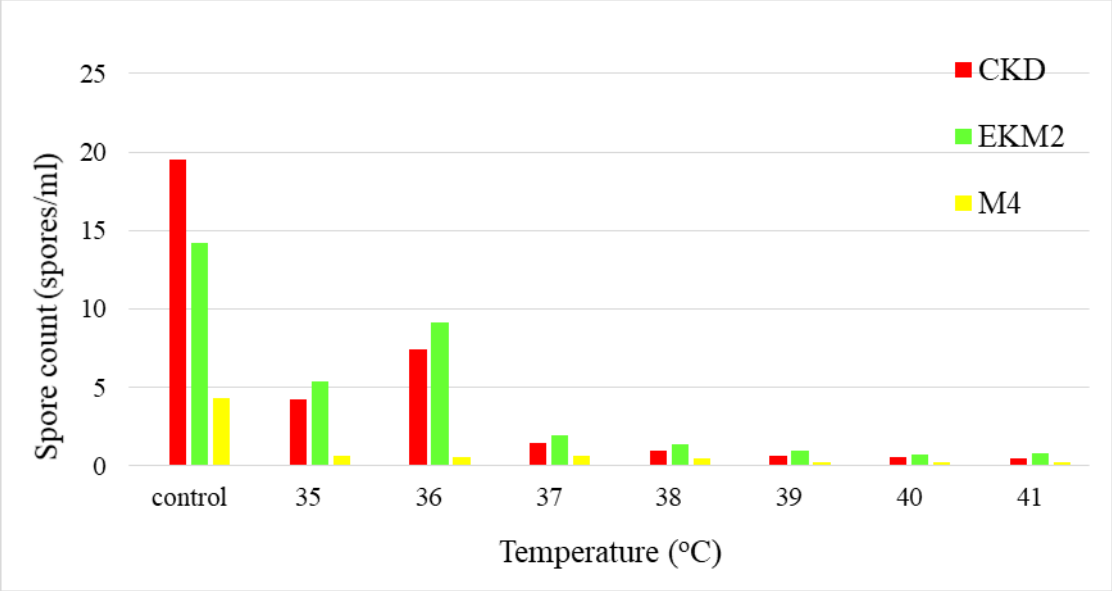


Fig 9.Effect of induced temperature on spore count of *Metarhizium anisopliae* isolates



5.5.5. Effect of induced temperature on size and shape of the spores of *Metarhizium anisopliae* isolates

Maximum spore size (8.03 µm) was recorded for the isolate EKM2 at 35^{o}C and it was followed by CKD (7.35 µm) and M4 (6.08 µm). The isolate M4 had the least size of spores (1.98 µm) when the temperature was raised to 40^{o}C. At the same temperature, EKM2 had the longest spores (3.47 µm), followed by CKD (2.81 µm). At the highest induced temperature of 41^{o}C, EKM 2 recorded the longest spores (3.47 µm), followed by CKD (3.18 µm), and the isolate M4 had the smallest spores (1.63 µm). The CKD isolate exhibited oblong, oval, and dumbbell-shaped spores at 35°C. The EKM2 spores have an elongated, oval shape with two edges segmented. The isolate M4 has spores that are longer, elongated, oval, and have thick walls. Compared to earlier treatments, the spore size reduced when temperature was raised at 40^{o}C. The spores of CKD and EKM 2 are smaller, abruptly formed, and had thicker walls. All the isolates had very small, shrunken, spores with thicker walls that clustered together at 41°C. Ekbom (1979) reported that the isolates of *M. anisopliae* with conidiospores had an average size of 6.1 µm and 5 µm. Friederichs (1920) had also noted the existence of a long-spored form of *M. anisopliae* with conidia 9-14µm long and a short-spored form with conidia 6-8 µm long.

With increase in temperature spore size of EKM2 and M4 showed a reducing trend. The isolate CKD also recorded reduced spore size with increase in induced temperature up to 37^{o}C and after that there was a twofold increase in spore size at 38^{o}C (3.197 to 6.747 µm). After that up to 40^{o}C, CKD showed a reducing trend, but at 41^{o}C again there was an increase in spore size (2.81 to 3.18 µm) denoting its plasticity to adapt with varying temepratures. At all induced temperature levels, the isolates EKM2 recorded significantly higher spore size when compared to the other two isolates indicating its capacity to tolerate temperature extremes. As a result, EKM2 has been identified to be thermotolerant, M4 has shown to be susceptible to temperature stress, and CKD has been revealed to be slightly temperature tolerant (Fig 10).

5.5.6. Effect of induced temperature on mycelial thickness of *Metarhizium anisopliae* isolates

At all temperatures except at 37ºC, the isolate EKM2 recorded significantly highest myceial thickness when compared to the other isolates. The isolate EKM2 has the thickest of mycelia (7.16 µm) at 35°C, followed by CKD (5.24 µm) and M4 had the thinnest mycelia (3.68 µm). EKM2 displayed a mycelial thickness of 3.27 µm at a temperature of 40 °C, followed by CKD (2.44 µm). When compared to EKM2, the isolate M4 had a less mycelial thickness of 2.47 µm.Maximum mycelial thickness of 3.07 µm was exhibited by the isolates EKM2 even at the greatest temperature of 41ºC, followed by M4 (2.65 µm) and CKD (2.59 µm), which were on par with each other. The findings indicated that at all the temperature levels, EKM2 has recorded a greater mycelial thickness and isolate M4 has demonstrated low mycelial thickness compared to EKM2 and CKD. The study showed that isolate EKM2 is superior to other isolates CKD and M4. (Fig 11). Glare *et al.,* (1996) pointed out that the phialides ranged from 11.4 x 2.6 µm on PDA to 9.0 x 2.9 µm on SDYA and 7.5 x 2.1 µm. Mongkolsamrit *et al.,* (2020) has also found that *Metarhizium* spp. have phialides which are smooth-walled, ovoid, occasionally subglobose and cylindrical with size of 4.5–6.5 × 1.5–2 µm.

5.5.7. Maintaining the thermotolerant isolates for six generations

The selected thermotolerant isolate EKM2 was grown at 41ºC continuously for up to six generations to confirm its temperature tolerance. The isolate EKM2 was shown considerable amount of growth and mycelial thickness at all the six generation with higher sporulation. Hence the isolate CKD has confirmed its drought tolerance.

5.6. Inducing drought tolerance in *Metarhizium anisopliae* isolates

Due to increasing temperature, the moisture stress has increased in the field now a days. The entomopathogenic fungi have to grow at lower moisture levels in the field conditions for successful biological control. The *M. anisopliae* isolates CKD, EKM2 and M4 has been grown continuously at increasing levels of PEG concentrations in order to induce drought tolerance.

Fig 10. Effect of induced temperature on spore size of *Metarhizium anisopliae* isolates

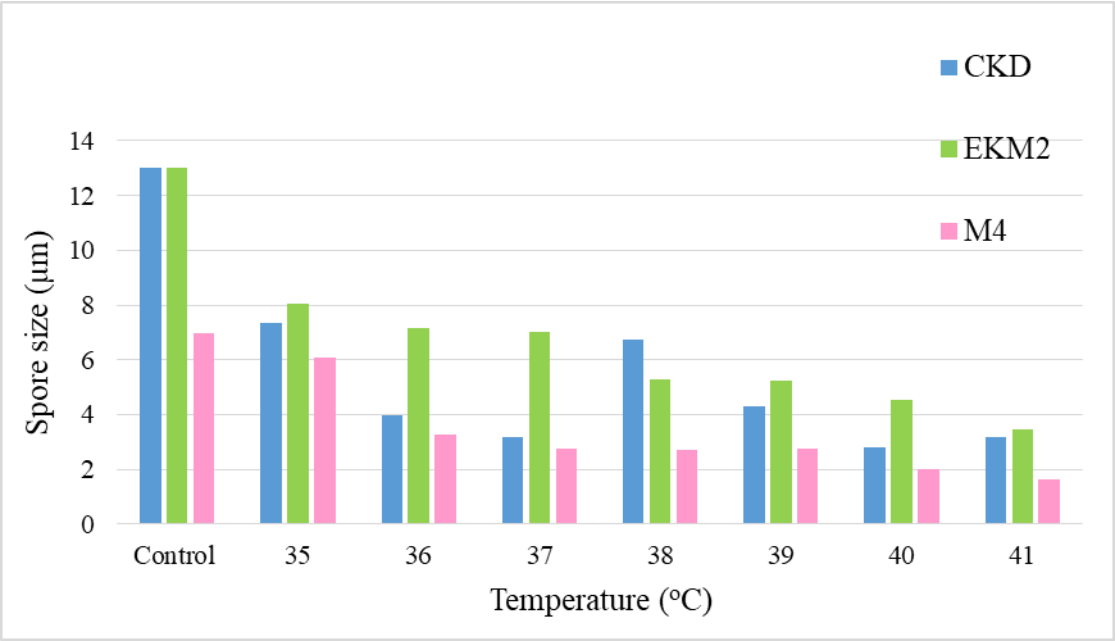
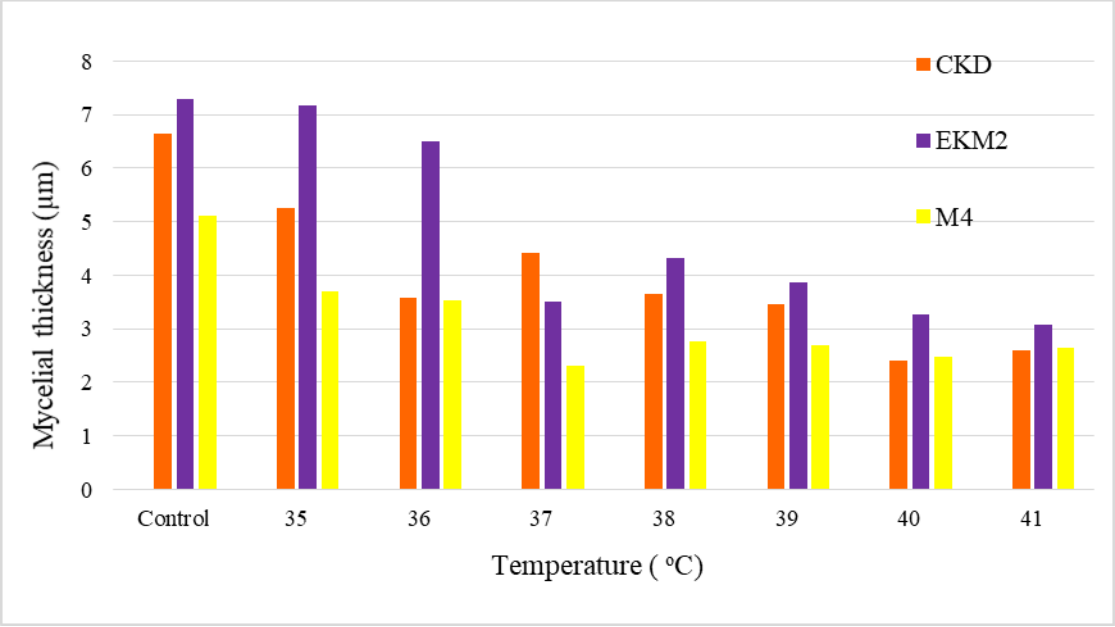


Fig 11. Effect of induced temperature on mycelial thickness of *Metarhizium anisopliae* isolates



5.6.1. Effect of induced drought on mycelial weight of *Metarhizium anisopliae* isolates

At 25% PEG concentration the isolate CKD had the highest mycelial weight (3.40 g), followed by EKM2 (2.80 g), while isolate M4 had the lowest mycelial weight (1.86g). The isolates, CKD recorded a greater mycelial weight of 0.95 g when the PEG content was raised to 38%, followed by EKM2 with 0.65 g. At 38% of the PEG concentration, the isolate M4 has not exhibited any growth. A greater mycelial biomass of 0.85 g was recorded by CKD when PEG content was increased to 39% and it was followed by EKM2 with a weight of 0.51 g. Isolates have grown at a maximum PEG concentration of 40 per cent and at the same PEG concentration, CKD has exhibited a mycelial weight of 0.62 g, followed by EKM2 with a weight of 0.38 g. None of the isolates had shown growth at 41per cent PEG concentration, so the experiment was terminated at this point. The findings showed that at all the PEG concentrations except at 37 per cent, the isolate CKD registered significantly highest mycelial weight when compared to the other isolates. Therefore, isolate CKD has proven to be the best of the three isolates and it is considered as drought-tolerant. The isolate EKM2 displayed moderate sporulation and mycelial weights ranging from 0.380 g to 2.890 g. The isolate M4 had the lowest weight of mycelia, had very little sporulation at all PEG concentrations, and had no growth after 37 per cent PEG. The isolate M4 is found to be vulnerable to drought (Fig. 12). Humphreys *et al.*, (1989) found that, the decrease in the yield of *M. anisopliae* biomass observed at 0.7 M-PEG was probably due to the inhibitory effect PEG has on growth. The white muscardine fungus, *B. bassiana* conidia germinated either on nutrient agar adjusted to 0.90 aw and exposed to a corresponding relative humidity or in liquid medium at 0.92 aw (Fargues, 1992). Hallsworth and Magan (1988) found that *M. anisopliae* and *B. bassiana* inoculated on SDA medium developed at 0.93 aw.

5.6.2. Effect of induced drought on spore germination of *Metarhizium anisopliae* isolates

The isolate CKD had the largest number of colonies at 25 per cent PEG concentration (92 X 10^3 CFU/ ml), followed by EKM2 (82 X 10^3 CFU/ ml) and the isolate M4 had the fewest colonies overall (59 X 10^3 CFU/ ml). At higher concentrations of PEG (39%), the isolate CKD produced 2 X 10^3 CFU/ ml followed

by EKM2 with 1 X 10^3 CFU/ ml. When the PEG concentration was enhanced to 40 per cent, maximum number of colonies was produced by CKD (4 X 10^3 CFU/ ml) and was followed by EKM2 with 0.66 X 10^3 CFU/ ml. From the results, it is evident that a higher spore germination was observed in the isolate CKD at all levels of PEG concentration followed by EKM2. The least CFU/ml was recorded by isolate M4. Hence, the isolate CKD was identified to be tolerant to drought and M4 was found to be a susceptible isolate. The isolate EKM2 was found to be moderately tolerant to drought (Fig 13). This is in similarity with the findings of Leucona *et al.*, (1997), where they observed that, *B. bassiana* conidia germinated faster at a water activity of 0.93 than *M. anisopliae* conidia and even at the unfavourable 0.93 aw, most isolates of both fungal species completed germination within 216 h, except *M. anisopliae* IP 225 where no germination was detected. The findings of Gillespie and Jones (2002) indicate that, *M. anisopliae* strains germinated generally more quickly than those of *B. bassiana* strains at water activities of 0.90–0.96.

5.6.3. Effect of induced drought on spore count of *Metarhizium anisopliae* isolates

The number of spores per millilitre (spores/ml) was calculated from the spore counts of all the three isolates at all PEG concentration levels. From the Fig. 11 it is clear that at all the PEG concentration levels, except at 39 per cent the isolate CKD registered significantly superior spore count when compared to other isolates. Isolate CKD had highest spore count of 15X 10^7/ml at 25per cent PEG, while EKM2 had 6.3X 10^7/ml and M4 was the one with the lowest spore count of 1.73X 10^7 /ml.The spore counts for the isolates CKD and EKM2 at 38% PEG were equal at 0.40 X 10^7 /ml. At 38 per cent PEG, the isolate M4 did not exhibit any growth. At 39 per cent PEG concentration, EKM2 had a higher spore count (0.46 X 10^7/ml) than CKD (0.33X 10^7/ml). The isolate CKD registered the highest spore count (0.323X 10^7/ml) at the highest PEG concentration of 40 per cent, followed by EKM2 (0.23X 10^7/ml). The findings indicated that at all PEG levels, CKD recorded a higher spore count per millilitre of PD broth and the isolate M4 had the fewest spores. Thus, it is concluded that CKD is a drought-tolerant isolate, EKM2 is a moderately tolerant one, and M4 is the most sensitive isolate with least spore count at all the concentrations of PEG. (Fig 11).This is similar to the study of Borisade and Magan (2014) who identified that water stress (0.98–0.94 aw) significantly reduced sporulation of all the fungal strains. Gervais *et al*. (1988), also showed that *Pencillium roquefortii* strains from cheese grew optimally

at 0.97- 0.98 aw, while maximum spore production was at 0.96 aw. Judet *et al.*, (2008) shown that the effect of a reduced water activity during sporogenesis on the germination of *P. chrysogenum* was greater at 0.95 Wa than at 0.99 Wa.

5.6.4. Effect of induceddrought on size and shape of spores of *Metarhizium anisopliae* isolates

In order to know the influence of moisture stress on spores, spore size and spore shape were analysed for all the three isolates under varying PEG concentrations. EKM2 has demonstrated a longer spore length of 9.22 m at 25 per cent PEG concentration, followed by CKD with a length of 8.32 µm. The lowest spore size was detected in the isolate M4 (6.43 µm). The spore length of EKM2 was 2.45 µm at 38 per cent of PEG, while CKD resulted in a significantly higher spore size of 3.15 µm. The isolate M4 did not exhibit any spores after 37 per cent of PEG concentration. In CKD, a greater PEG concentration of 39 per cent led to longer spores with a length of 2.68 µm, followed by EKM2 with a length of 2.46 µm. When subjected to a peak PEG concentration (40%), both EKM2 and CKD recorded the longest spores (2.39 µm and 2.27 µm respectively) and were on par with each other.

Significant variations in the spore shapes were observed for all the isolates at different moisture stress conditions. At 25 per cent of PEG, EKM2 produced long, oval spores with segmentation at both edges while CKD showed elongated, oval, big spores. The isolate M4 showed long, oval, thick-walled spores at the same concnetration. At 38% of PEG, CKD has displayed small, long, oval-shaped, and shrivelled spores, and EKM2 has displayed small, oval to round, shrinking spores. At 39 per cent of PEG, CKD produced oval, shrunken, small-sized spores, while abrupt-shaped, small-sized spores were observed in EKM2. When moisture stress is at its peak (40 % PEG), CKD has produced very tiny, round spores with thick walls, and EKM2 has produced tiny, narrow spores that were shrunken. The findings indicate that the drought tolerance of CKD which produced the longest spores at almost all PEG concentrations (Fig 12). This is accordance with the findings of Driver *et al.*, (2000) where they reported that, conidia of *M. anisopliae var. lepidiotum* have a spore length in the range 7.3 to 10.6 µm and a width of 3 to 4.1 µm. Rombach *et al.*, (1987) has found that *Metarhizium var. minus* isolates with smaller swollen conidia, 4.5-7.0 x 2.03-3.0 µm, found on plant hoppers in the Philippines and Solomon Islands.

Fig 12. Effect of induced drought on mycelial weight of *Metarhizium anisopliae* isolates

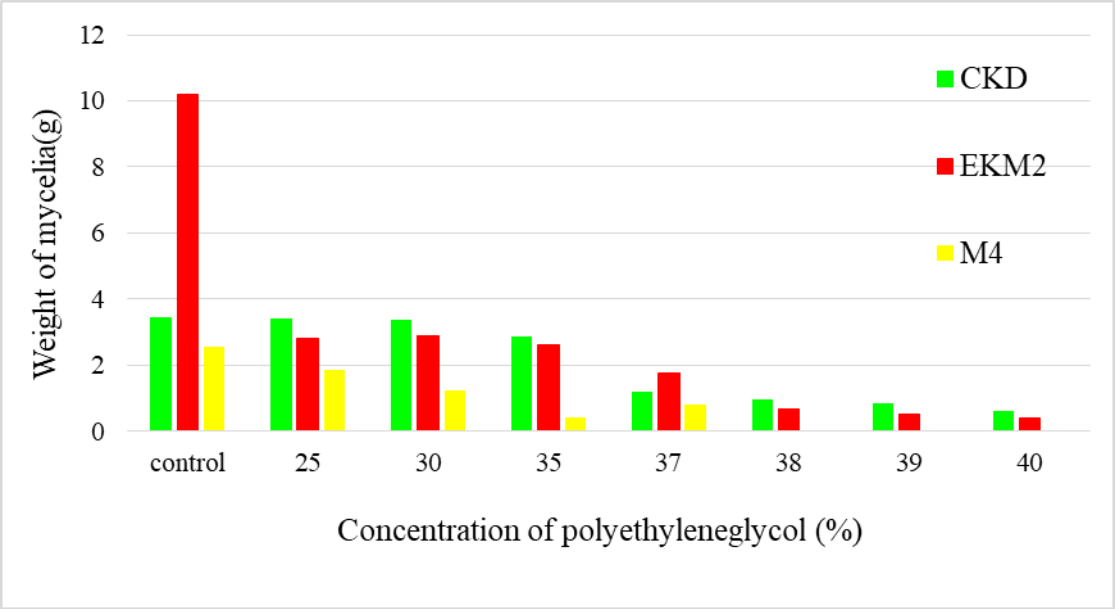
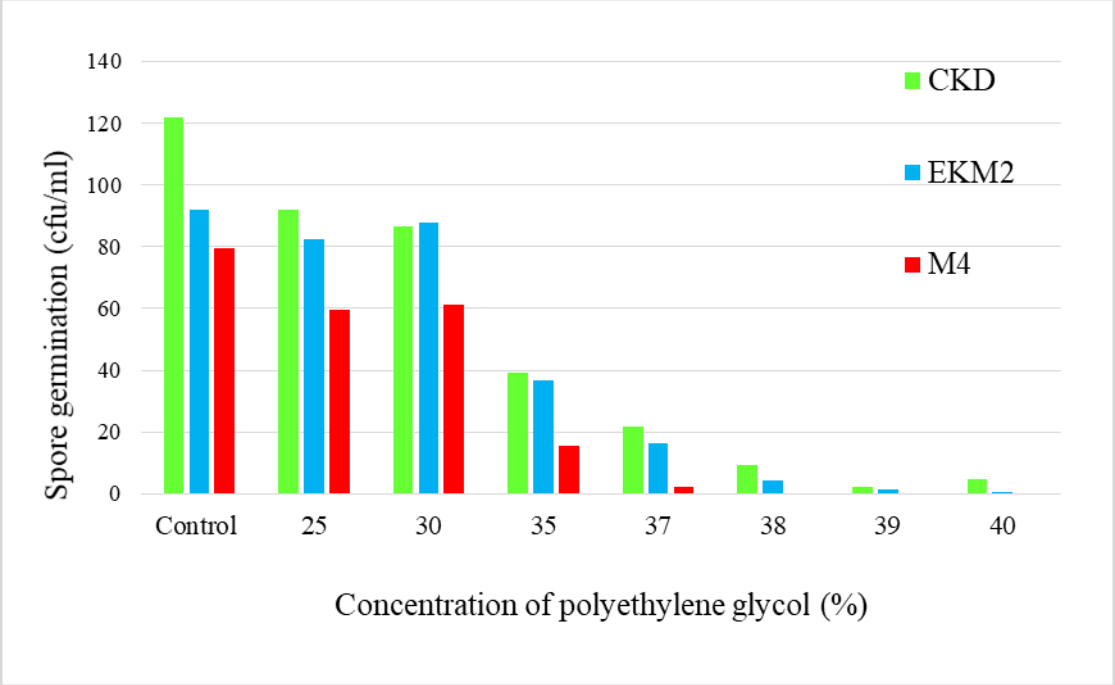


Fig 13. Effect of induced drought on spore germination of *Metarhizium anisopliae* isolates



5.6.5. Effect of induced drought on the mycelial thickness of *Metarhizium anisopliae* isolates

Mycelial thickness of all the isolates were examined under different moisture stress conditions in order to know their survival capacity under drought conditions. As per Fig. 13 it is clear that spore size of all the three isolates are showing a reduced trend with increase in PEG concentrations. At all the moisture stress levels, except at 25 per cent PEG, the isolate CKD recorded significantly superior spore size when compared to the other isolates. The isolate CKD (4.79 µm) had the thickest mycelia at 25% PEG concentration, followed by EKM2 (3.91 µm) and M4 recorded the thinnest mycelia (2.72 µm). At 39 per cent PEG, the isolates CKD and EKM2 both had mycelial thickness measurements of 2.58 µm and 3.45 µm, respectively. At the highest PEG concentration of 40 per cent, isolate EKM2 and CKD recordedthe thickest mycellial layer (2.72 µm and 2.53 µm respectively) and were on par with each other. The findings showed that at all temperature settings, CKD had a larger mycelial thickness. The isolate M4 recorded a thinner mycelium. It is evident from the Fig. 13 that the isolate CKD performed well under different moisture stress conditions with significantly superior mycelial thickness except at 35 per cent PEG concentration. At 35 per cent PEG concentration, the isolate EKM2 recorded slightly higher mycelial thickness (3.167 µm) when compared to CKD (2.713 µm) and M4 (2.590 µm). The isolate M4 could not survive higher PEG concentrations starting from 38 per cent PEG.

This is in agreement with Glare *et al.*, (2012) who found that the average phialide dimensions of *M. anisopliae* are in the range 8-12 x 2.4-2.8 µm. As per Gams and Rozsypal (1973), *M. Flavoviride* have more swollen conidia measuring 7.0-11.0 x 4.5-5.5 µm borne on "club-shaped" phialides, with a strongly tapering and thick-walled conidiogenous apex.

5.6.6. Maintaining the drought tolerant isolates for six generations

The selected drought tolerant isolate CKD was grown at 40 per cent PEG continuously for up to 6 generations to confirm its drought tolerance. The isolate CKD has shown considerable amount of growth and mycelial thickness at all the six generation with higher sporulation and hence confirmed its drought tolerance.

Fig 14. Effect of induced drought on spore count of *Metarhizium anisopliae* isolates

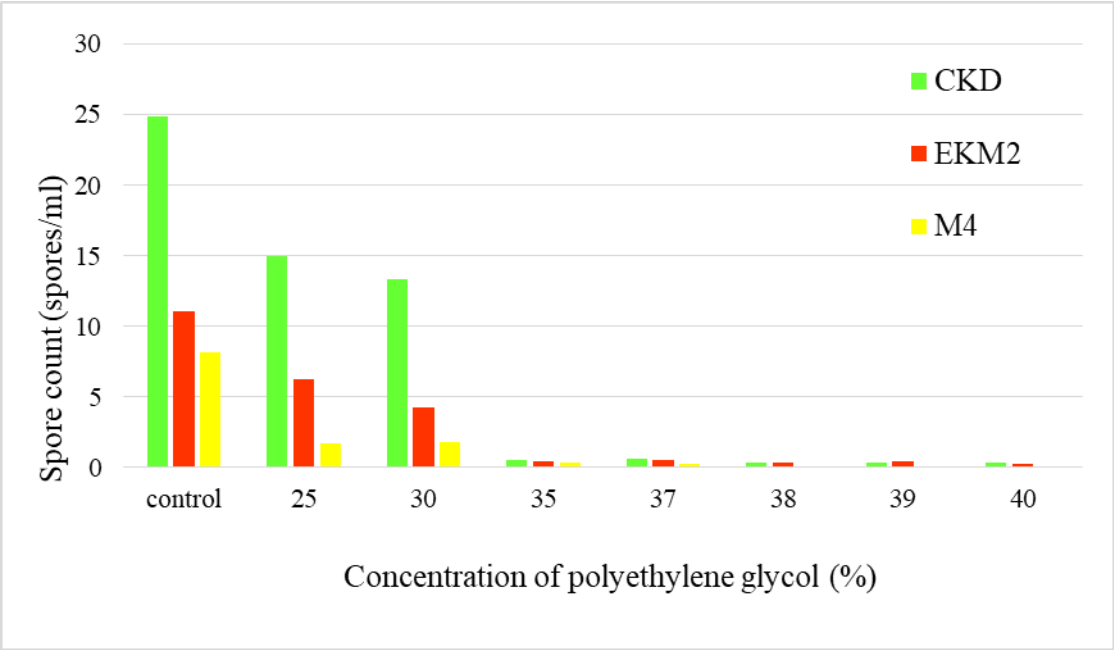


Fig 15. Effect of induced drought on size and shape of spores of *Metarhizium anisopliae* isolates

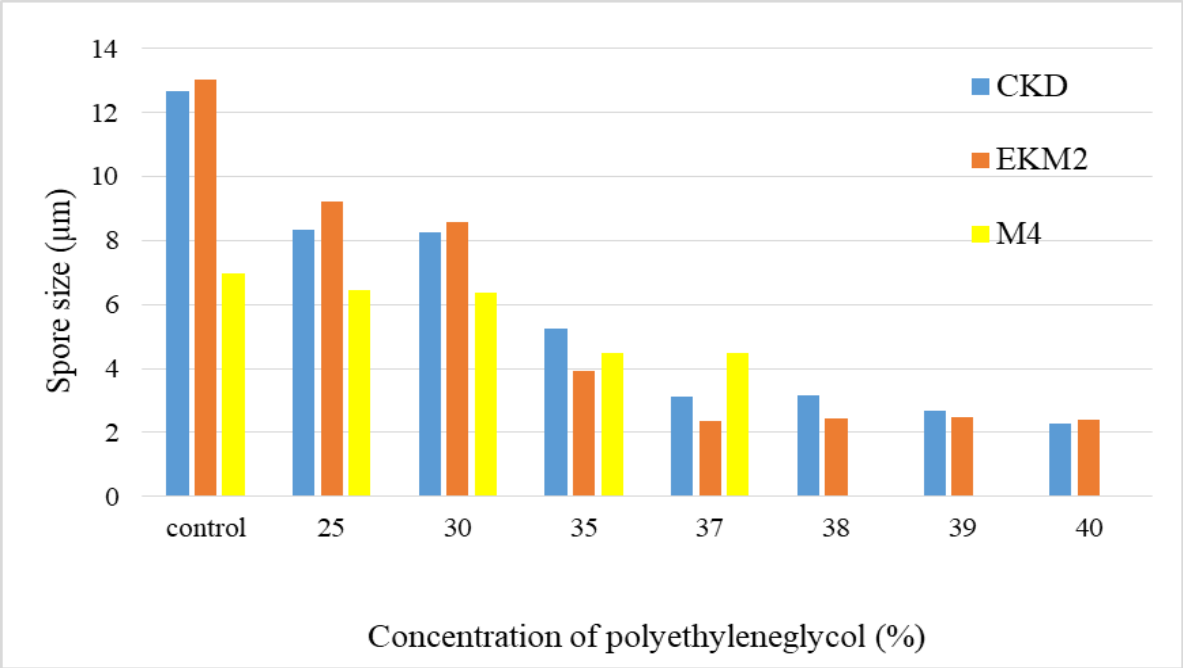
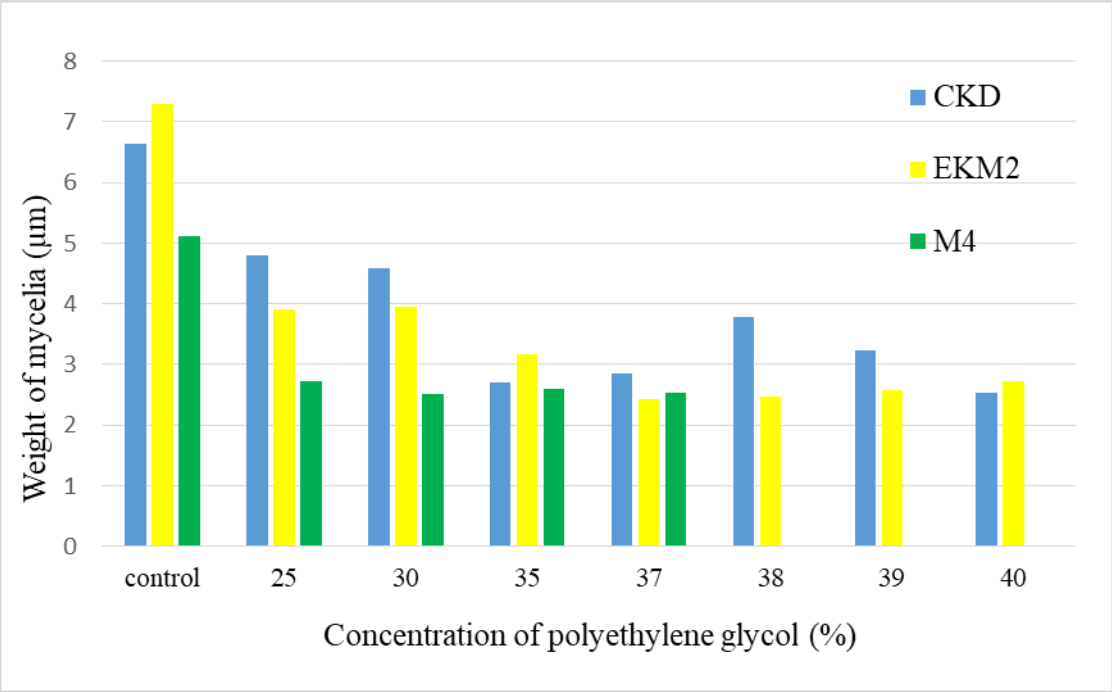


Fig 16. Effect of induced drought on the mycelial thickness of *Metarhizium anisopliae* isolates



5.7. *In vitro* screening of *Metarhizium anisopliae* Sorokin isolates for biocontrol efficacy

The selected tolerant isolates were tested for their bioefficacy on *Galleria mellonella* larvae for 10 days (Fig 17).

5.7.1. Per cent mortality of *Galleria mellonella* larvae

Up to three days after treatment, none of the isolates caused larval mortality at any concentrations. From the fifth day onwards, every isolate began to exhibit mortality. The isolate CKD showed mortality at concentrations ranging from 26.66 to 73.33 per cent on the fifth day at 10^7 to 10^9 spores/ml. On the fifth day, EKM2 demonstrated a death rate of 26.66 per cent at 10^8 spores/ml and 33.33 per cent at 10^9 spores/ml. Additionally, M4 has also demonstrated a mortality of 20 and 26.66 per cent at concentrations of 10^8 and 10^9spores/ml, respectively. At 10^5 spores/ml, CKD recorded mortality of 43.33 per cent, while EKM2 had a higher mortality of 66.66 per cent, and M4 has recorded a mortality of 33.33 per cent. At nine days of treatment, at 10^8 spores/ml, cent per cent mortality was recorded by the isolates EKM2 and it was immedietly followed by CKD with 96.66 per cent mortality. The isolate M4 also recorded more than 80 per cent mortality. Both CKD and EKM2 have exhibited cent per cent mortality at the maximal dosage of 10^9 spores/ml, while M4 has showed 86.66 per centmortality. Compared to CKD and M4, EKM2 had a greater mortality rate at all doses by the ninth day (Fig 17). This is similar to the findings of Serebrov, (2006) who reported that, isolates of both *B. bassiana* and *M. anisopliae* caused 86.67 to100 per cent mortality on *G. mellonella*. Khalid *et al.,* (2011) also reported that, high virulent isolates showed comparable efficiency with *B. bassiana* and *M. anisopliae* that caused cent per cent and 98.4 per cent mortality of *G. mellonella* larvae, respectively at 10 days post-application with the concentration of 1 x 10^8 spores/ml.

The findings proved that EKM2 and CKD caused mortality rates at all spore concentrations. In comparison to higher doses, the mortality was quite low at 10^5 and 10^6 spores/ml. On the ninth day of treatment, only EKM2 exhibited cent percent mortality at doses of 10^8 and 10^9 spores/ml. Therefore, when compared to CKD, EKM2 had proved to be a better isolate. Compared to M4, isolate CKD has demonstrated a greater mortality rate. This is in conformity with the study of Ferron

(1978), who reported that temperatures lower than optima retard the development with out necessarily affecting the total mortality. The susceptible isolate M4 has also demonstrated a considerable mortality rate. Study conducted by Habtegebriel *et al.,* (2016) showed that, native isolates in Ethiopia recorded high mortality of 71.3 per cent by *Beauveria* spp. and 75 per cent with *Metarhizium* spp. at 1 x10^8 spores/ml of 10 days post-application. The study of Nderiyamana et al. (2019), clearly indicated that some of the isolates were effective as the commercially formulated Metarhizium that caused 82.8 per cent larval mortality of *Tuta absoluta* at 10^8 spores/ml.

5.7.2. Dose – mortality responses of *Galleria mellonella* larvae

Following treatment with isolates of *M. anisopliae*, the lethal time (LT_{50} and LT_{90}) for mortality of *Galleria* larvae was estimated. Treatment dosages of 10^5 to 10^9 spores/ml were utilized on the susceptible isolate and the selected tolerant isolate.The isolate EKM2 had the shortest LT_{50} (7.989 days), followed by CKD (8.806 days), and isolate M4 had the longest LT_{50} (9.46 days) at the lowest concentration of 1x 10^5 spores/ml. EKM2 (10.788 days) and CKD had the lowest LT_{90} values at this concentration and M4 has taken highest LT_{90} (14.097 days). The isolate EKM2 had a shorter lethal time for *Galleri*a larvae than other isolates at the lowest dosage of 1x 10^5 spores/ml, and it was followed by CKD. The isolates used for treatment had a maximum concentration of 1x 10^9 spores per ml. The isolate CKD has a lower LT_{50} value (5.099 days) at the highest dosage of 1 X10^9 spores/ml followed by EKM2 (5.359 days). The isolate M4 had an LT_{50} of 6.124 days, which was the highest figure. The isolate CKD (6.780 days) and EKM2 had the lowest LT_{90} values at this dosage. The isolate M4 had the highest LT_{90} value, which was 8.788 days. At the highest spore concentration of 1x 10^9 spores/ml, the isolate CKD showed the lowest time for the 50 and 90 per cent mortality of the *Galleria* larvae (Fig 18). This can be related to the findings of Bischoff *et al.,* (2009) who found that the median lethal time (LT_{50}) of isolates of *B. bassiana* and *M. anisopliae* differed among isolates with the range between 2.36 -5.01 days. Ibrahim *et al.,* (2016) has also reported that isolates of *B. bassiana* triggered high mortality of *G. mellonella* larvae within the shortest time of 2.2 and 2.3 days.

The results showed that when the spore concentration was increased, both LT_{50} and LT_{90} values decreased. Lower LT_{50} and LT_{90} values have been observed for isolate CKD at the highest dose of 1x 10^9 spores/ ml. The isolate EKM2 has also recorded the lower LT50 and LT90 values at all other dosages. At all the concentrations spores, the

isolate M4 recorded the greatest LT_{50} and LT_{90} values. Therefore, when used at a higher concentration, EKM2 and CKD are demonstrated to be elite and virulent isolates, whereas M4 is discovered to be less virulent than the other two isolates (Fig. 18).

This is in conformity with the findings of Kaur and Padmaja (2008) who categorized the entomopathogenic fungal isolates with LT_{50} value < 5 day as highly virulent, between 5-6 days as moderately virulent and >6 days as less virulent. Ndereyimana *et al*. (2019) and Tadele and Emana (2017) reported a lower lethal time for *Metarhizium* with LT_{50} of 3.9 days against the larvae of T. absoluta at a spore concentration of 10^8 spores/ml in Rwanda and 5.21 days with 2.5 x 10^8 spores/ml of *Metarhizium anisopliae* in Ethiopia, respectively.

5.8. Biochemical analysis of stress tolerance and virulence

Microorganisms including entomopathogenic fungi will exhibit different kinds of defense mechanisms in order to tolerate stress conditions. The increased rate of enzyme activity is also contributing to their virulence. In this experiment, the influence of induced thermal and water stress on the activity of catalase, peroxidase, protease, lipase and chitinase as well as the variations in the amount of trehalose and total protein content were estimated.

5.8.1. Total protein content in stress-tolerant isolates of *Metarhizium anisopliae*

In contrast to the isolates cultivated at room temperature (26±2 ^oC), which had a total protein level of 0.215 mg/ml, the thermotolerant isolate EKM2 had a total protein content of 0.354 mg/ml. The drought-tolerant isolate (CKD) was found to contain 0.373 mg/ml of total protein. When grown at room temperature, the isolate CKD showed a protein content of 0.259 mg/ml, which is noticeably lower than the tolerant one (Fig 19 and Fig 21). This is in tune with the findings of Hohmann (2002) and Wang *et al.,* (2004) who found that, heat shock proteins can protect cells in response to various external stresses and the 20S proteasome subunit can help in the formation of appressoria. Ying (2006) reported that, a non- hydrophobic cell wall protein (CWP15) from B. bassiana was confirmed to be responsible for atleast 25 per cent of conidial tolerance to thermal stress at 48^oC. The abundance of HSP and other stress-related proteins may enhance conidial ability to survive environmental stresses (Cooper *et al.,* 2006).

Fig 17. Per cent mortality of *Galleria mellonella* larvae

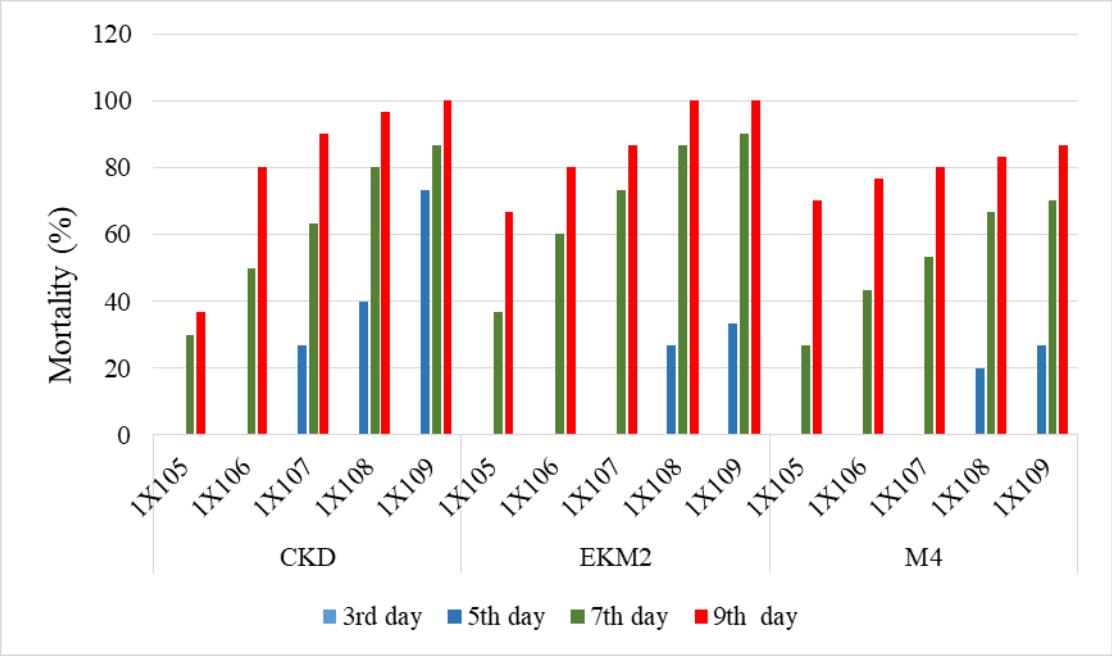
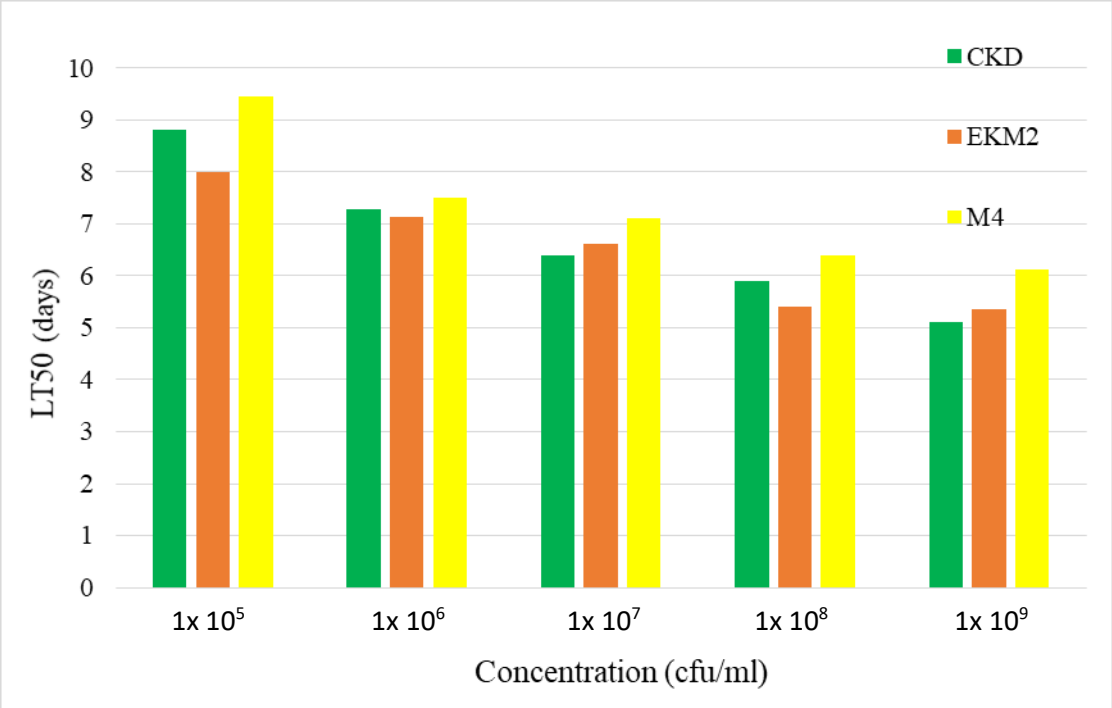


Fig 18. Dose- mortality responses of larvae of *Galleria mellonella* with *Metarhizium anisopliae*



5.8.2. Trehalose content in stress-tolerant isolates of *Metarhizium anisopliae*

When compared to the trehalose level of the same isolate cultivated at room temperature (1.074 mg/min/g tissue weight), the thermotolerant isolate EKM2 had a trehalose content of 1.954 mg/min/g tissue weight. The drought-tolerant isolate CKD had a trehalose level of 1.970 mg/min/g tissue weight, which was substantially higher than the control (1.224 mg/min/g tissueweight) (Fig.20 and Fig. 22).This is in agreement with the study of Hallsworth and Magan (1996) who found that an increased intracellular trehalose concentration has been often associated with tolerance of heat shock. The degree of freeze tolerance acquired at 4°C closely correlates with the trehalose content and at 4°C, where the maximal levels of trehalose were generated and cells acquired the highest resistance to freezing (Kondror *et al.*, 2004). Virgilio *et al.*, (1991) and Nwaka *et al.*, (1995) has also reported that the increase of neutral trehalose activity during heat shock has been extensively studied in *Saccharomyces cerevisiae* and *S. pombe* and found to be mediated by both transcriptional and posttranslational regulation by cAMP-dependent protein kinase A (PKA)-mediated phosphorylation.

5.8.3. Activity of enzyme catalase and peroxidase in stress tolerant isolates of *Metarhizium anisopliae*

The thermotolerant isolate recorded higher catalase activity of 0.386 EU/min/mg protein than its control (0.237 EU/min/mg protein). The drought-tolerant isolate, CKD, had catalase activity of 0.384 EU/min/mg protein, but this activity decreased to 0.240 EU/min/mg protein for control. The results showed that the tolerant isolates have considerably higher catalase activity than their control (Fig 23 and Fig 24). Santi *et al.*, (2010) found that, catalase helps to protect *M. anisopliae* conidia from abiotic stress, and its expression is greater in mycelia, where it can protect against the cytotoxic effects of host-derived H_2O_2.

Fig 19. Total protein content of thermotolerant EKM2 *of Metarhizium anisopliae*

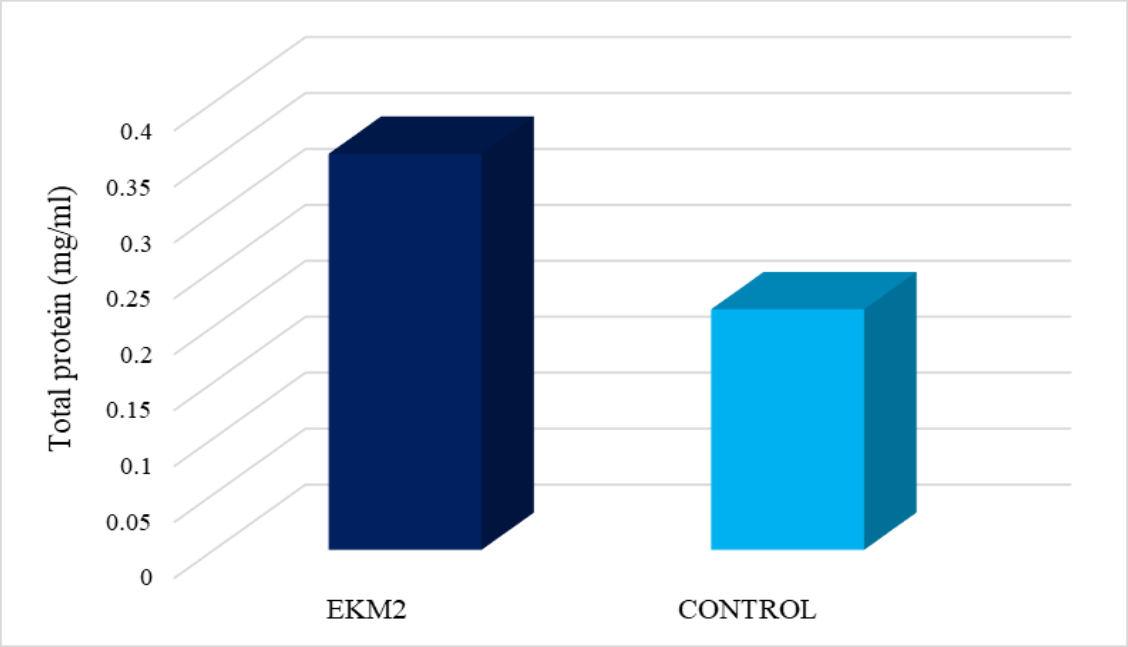


Fig 20. Trehalose content of thermotolerant EKM2 *of Metarhizium anisopliae*

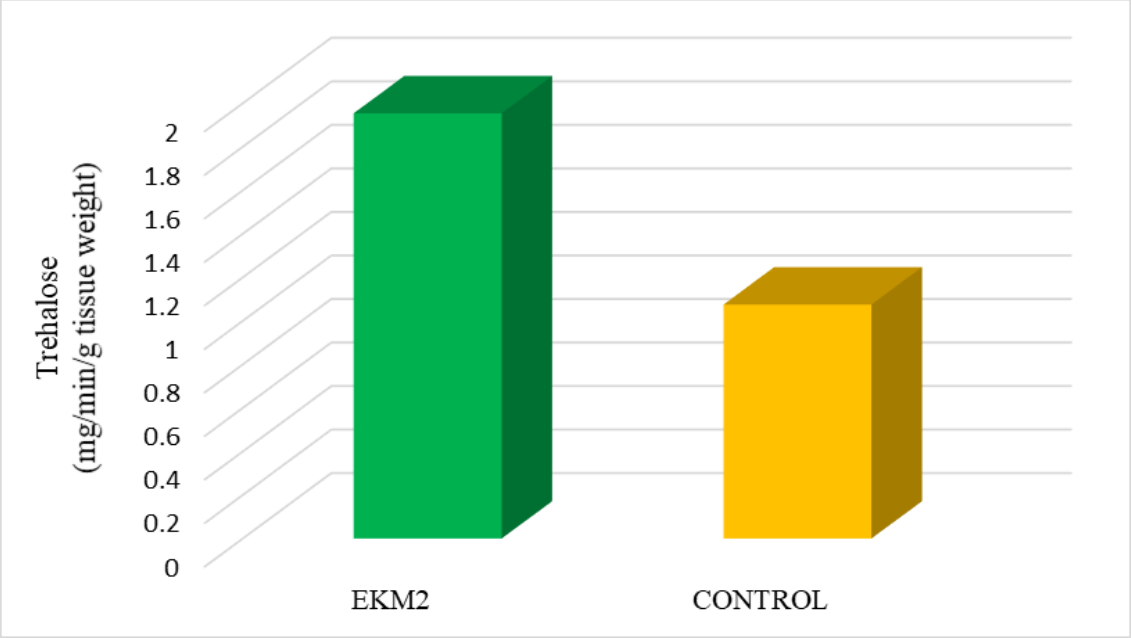


Fig 21. Total protein content of drought tolerant CKD *of Metarhizium anisopliae*

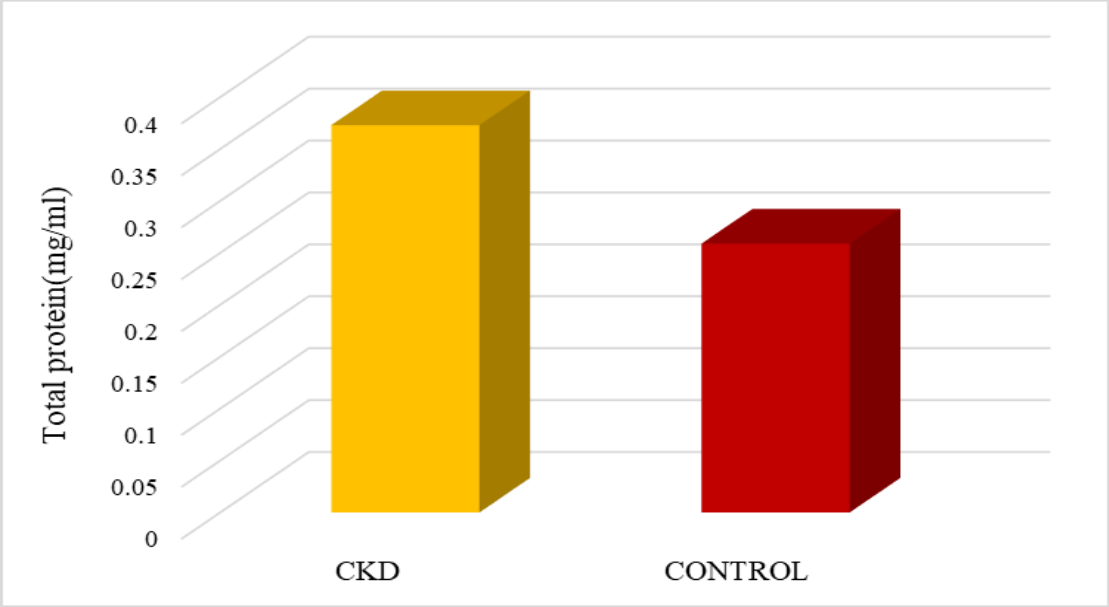
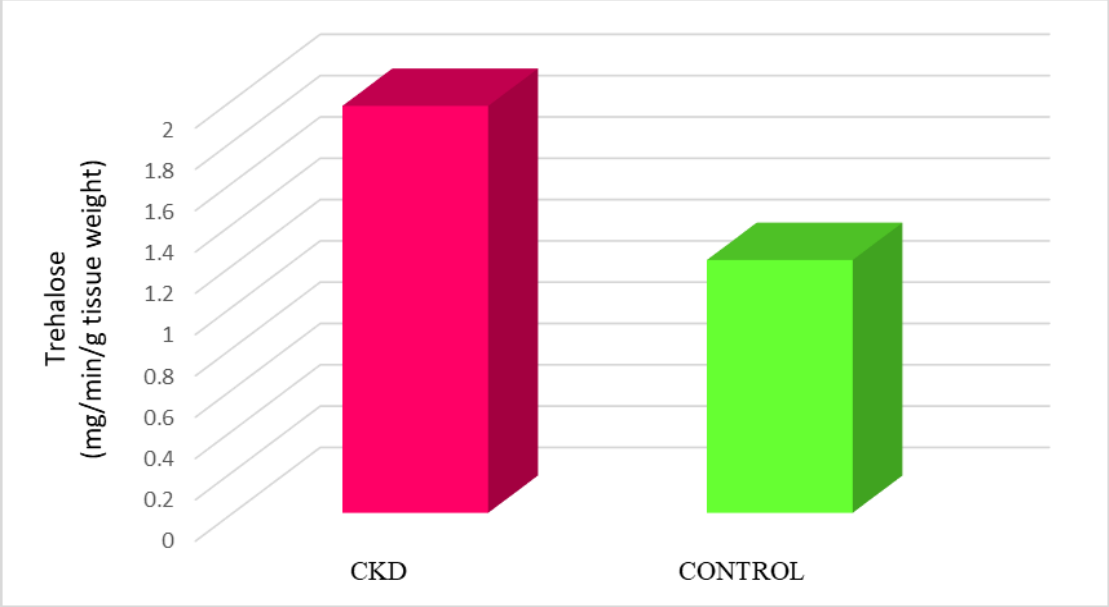


Fig 22. Trehalose content of drought tolerant CKD *of Metarhizium anisopliae*



The peroxidase activity of the thermo-tolerant isolate was 0.0230 EU/min/g tissue weight, which was noticeably greater than the control (0.0120 EU/min/g tissue weight). In contrast to its control, which had much reduced peroxidase activity (0.0130 EU/min/g tissue weight), the drought-tolerant isolate CKD had a peroxidase activity of 0.0236 EU/min/g tissue weight. The results indicate that the tolerant isolates have considerably higher peroxidase activity when compared to their respective controls (Fig 23 and Fig 24). This is in conformity with study of Anglova *et al.,* (2005) who showed that the activity of catalase was increased by 50–60 per cent under stress but for peroxide stress caused a 2-3 fold increase. Kapoor and Lewis (1987) has also indicated that heat shock and treatment with oxidative stress-inducing agents such as hydrogen peroxide and some metal ions enhanced peroxidase activity. The relative peroxidase activity was observed to be enhanced with increasing temperature up to 43°C, where a 2 fold increase in activity was apparent and at higher temperatures, there was a less pronounced increase, but even at 65°C, 1.5 foldhigher peroxidase activity was detected compared with that at 30°C (Machwe *et al.,* 2002).

5.8.4. Protease activity in stress tolerant isolates of *Metarhizium anisopliae*

In comparison to the control (0.467 EU/min/mg protein), the protease activity of the thermo-tolerant isolate EKM2 was greater (0.490 EU/min/mg protein). The protease activity of the drought tolerant isolate was 0.621 EU/min/mg protein, which was substantially higher than that of its control (0.483 EU/min/mg protein). The protease activity of stress tolerant isolates was much higher compared to the control (Fig.23 and Fig. 24). Shang *et al.,* (2023) reported that, the up regulation of proteases in the MrSkn7 mutant could trigger heavy melanisation responses in insects and thereby the production of melanins to attenuate fungal virulence. Braga *et al.* (1999) also reported that the proteolytic activities of *Metarhizium* strain were rapidly increased, reaching a maximum at about 5 days, when the highest production of biomass was also observed.

Fig 23. Enzyme activity of thermotolerant EKM2 isolate of *Metarhizium anisopliae*

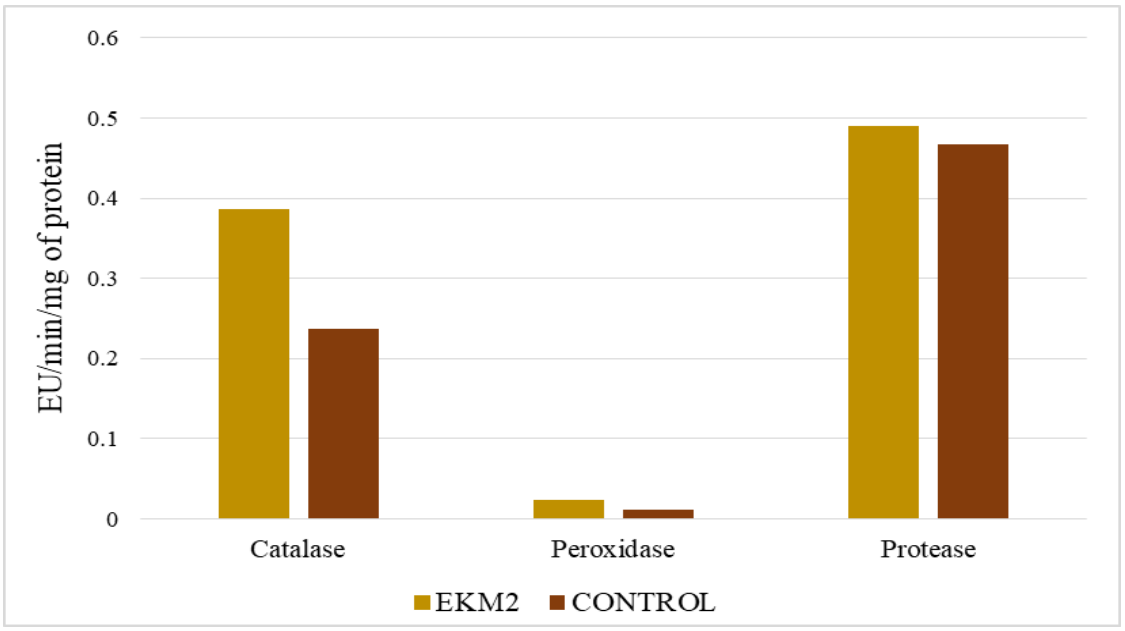
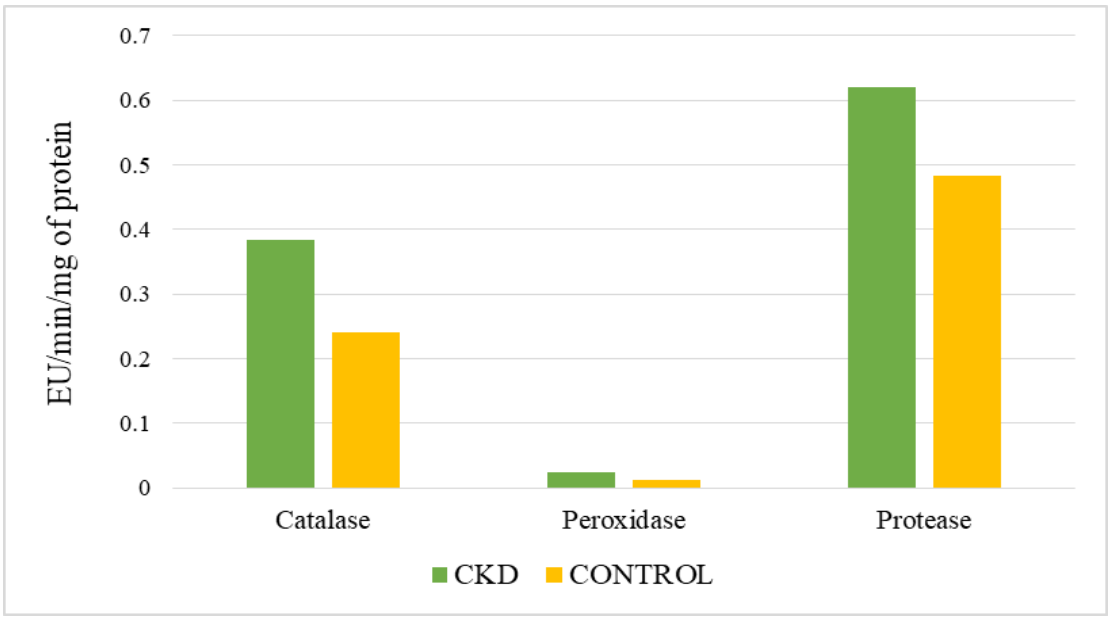


Fig 24. Enzyme activity of drought tolerant CKD isolate of *Metarhizium anisopliae*



5.8.5. Activity of enzyme lipase in stress tolerant isolates of *Metarhizium anisopliae*

The thermo-tolerant EKM2 exhibited lipase activity of 20.667 µmol fatty acid/ml after 5 min of the reaction, which is much higher than the activity of control (7.000µ mol fatty acid/ml). The thermotolerant isolate once again displayed greater lipase activity at 25 min of treatment (10.000 µmol fatty acid/ml) than the control (8.667 µmol fatty acid/ml). The level of lipase activity varied with time, which showed a peak at 5 min in case of the thermotolerant EKM2 and declining at 10, and then 15 min (Fig 25).

Both the drought-tolerant isolate and the control showed a lipase activity of 5.000 µmol fatty acid/ml after 5 min of the procedure. The drought-tolerant isolate has exhibited a lipase activity of 11.00µ mol fatty acid/ml at 25 min of the reaction, which was much greater than that of its control (5.667µ mol fatty acid/ml). The highest lipase activity of the drought tolerant isolate was observed at 25 min of the reaction followed by at 15 min. The findings are proving that stress tolerant isolates have a higher lipase activity than control (Fig 26). Hegedus and Khachatourians (1988) also studied the effect of temperature on the lipase production by *B.bassiana* and pointed out that, a growth temperature of 20°C produced substantially lesser amounts of lipase even though biomass production was higher.

5.8.6. Chitinase activity in stress tolerant isolates of *Metarhizium anisopliae*

On the first day, the chitinase activity of thermotolerant EKM2 was 1.607 g of N-acetyl glucose amine/min/ml of broth and that of the control was 1.140 g of N-acetyl glucose amine/min/ml. The control showed lowest chitinase activity (0.548 g of N-acetyl glucose amine/min/ml) on the fifth day, whereas the thermo tolerant EKM2 displayed higher activity of 1.140 g of N-acetyl glucose amine/min/ml of broth.

Fig 25. Lipase activity of thermo tolerant EKM2 isolate of *Metarhizium anisopliae*

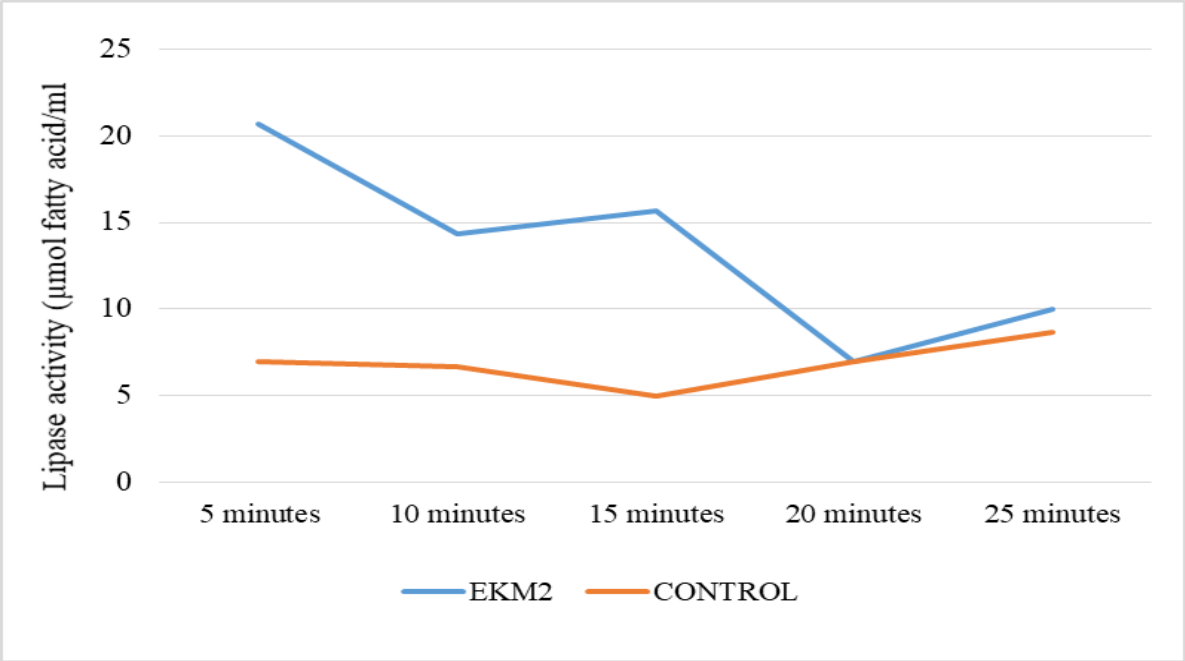
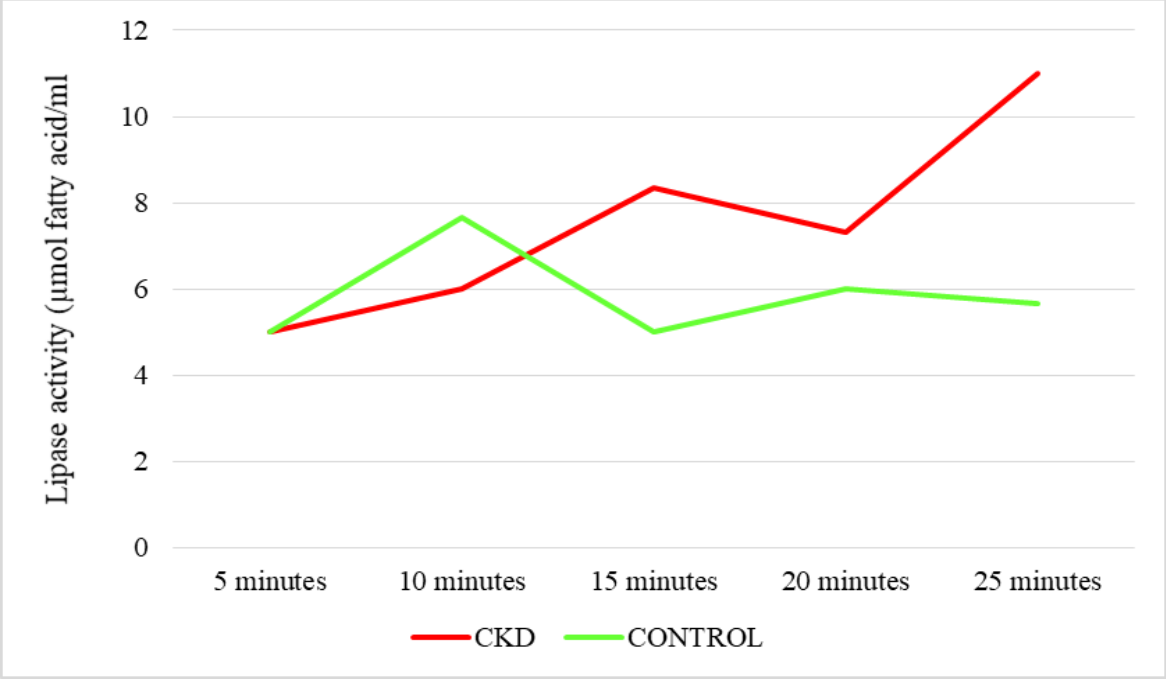


Fig 26. Lipase activity of drought tolerant CKD isolate of *Metarhizium anisopliae*



On the first day, the drought-tolerant CKD and its control had chitinase activities of 0.978 and 0.108 g of N-acetyl glucose amine/min/ml of broth, respectively. On the fifth day the drought-tolerant CKD recorded the highest chitinase activity (3.062 g of N-acetyl glucose amine/min/ml of broth) when compared to its control (0.074 g of N-acetyl glucose amine/min/ml of broth). The findings indicate that, in comparison to the control, the stress-tolerant isolates have increased chitinase activity (Fig. 27 and Fig. 28). This is in conformity with the study of Meena *et al.,* (2014) who found that, the enzyme activity of *M. anisopliae*was found to increase with temperature range up to 45°C then decreased sharply with further increase in temperature. Optimal chitinase production was reported at 30°C in *Streptomyces tendae*as reported by Kavitha and Vijaylakshmi, (2011) and at 45°C higher chitinase production was shown by*Bacillus licheniform* SK-1 and *Enterobacter sp*. NRG4 (Kudan and Pichyangkura, 2009). Samuels *et al.*, (1989) proved that highly pathogenic strains showed detectable amounts of extracellular chitinase, lipase and protease activities in the culture.

5.8.7. Protein profiling using SDS- PAGE

The protein profile using SDS-PAGE was done in order to identify the presence of heat shock proteins. The temperature induction had up regulated heat shock proteins of range 25 kDa to 35 kDa and expressed heat shock proteins 100 kDa and 180 kDa molecular weight in the isolate EKM2. This is confirming the thermo tolerance of the isolate EKM2. The drought induction on the other hand did not showed the presence of any heat shock protein in CKD when compared to the control. Both CKD and control had shown similar protein profiles and proved that drought did not cause expression of heat shock proteins.

Xavier and Khachatourians (1999) found that, heat shock proteins were identified in the insect pathogenic fungus *Beauveria brongniartii* after incubation at 45°C for 3 h. Several of the proteins were identified in *M. acridum* mycelia (HSP 70 and 90) conidia (Cooper *et al.* 2006; Leng *et al.* 2008). Cooper *et al.* (2006) has also found that, the abundance of HSP and other stress related proteins may enhance conidial ability to survive all types of environmental stress like temperature and drought.

Fig 27. Chitinase activity of thermo tolerant EKM2 isolate of *Metarhizium anisopliae*

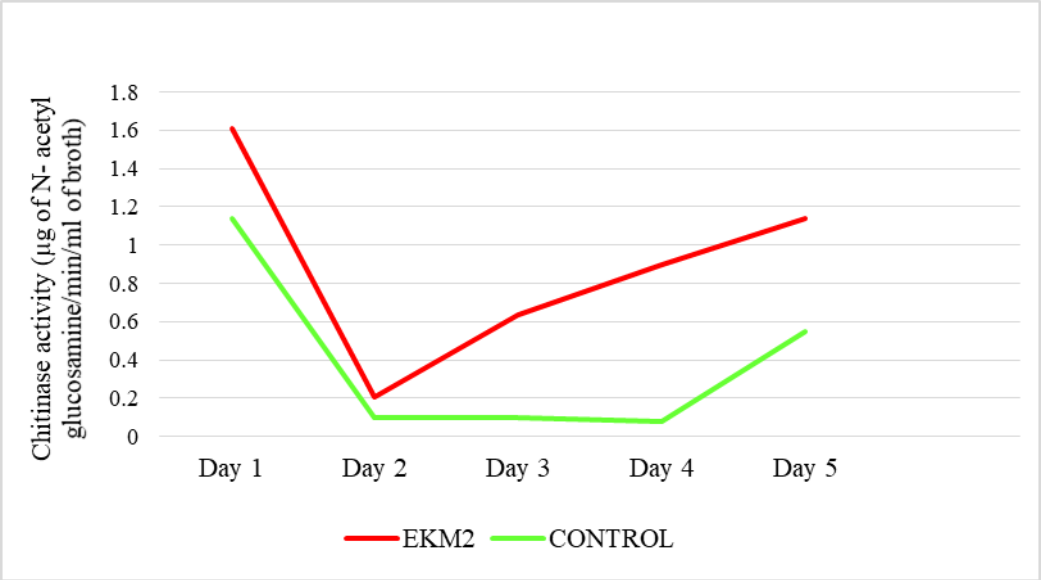
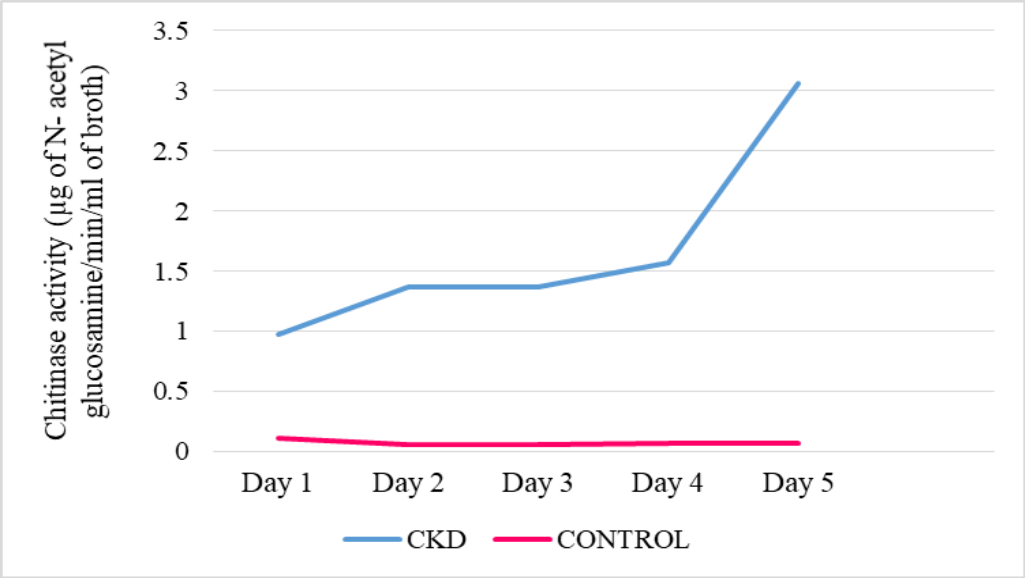


Fig 28. Chitinase activity of drought tolerant EKM2 isolate of *Metarhizium anisopliae*



SUMMARY

6. SUMMARY

Due to the rising concerns about the increased use of chemical pest control practices, entomopathogenic fungi are gaining importance as biocontrol agents for pest control. The green muscardne fungus, *Metarhizium anisopliae* is one among the EPF which are successful against wide range of insect pests. The major problem regarding the commercialization of *Metarhizium* sp. is the uncertainty in their efficacy under stress conditions in the field. Hence the study entitled "Novel strains of *Metarhizium anisopliae* Sorokin. (Ascomycota: Sordariomycetes) with enhanced abiotic stress tolerance" was conducted at the College of Agriculture, Vellanikkara, Thrissur during 2020-2022 for developing elite strains with improved stress tolerance. The study comprised of developing novel strains of *Metarhizium anisopliae* through induction of temperature and drought stress and also to evaluate the biocontrol efficacy against wax moth larvae (*Galleria mellonella*).

The striking findings of the study are summarized below.

- The existing isolates of *Metarhizium anisopliae* present at the Department of Agricultural Entomology, College of Agriculture, and Vellanikkara were characterized for their cultural, morphological, and molecular characters. The isolates were named as CKD, EKM2, and M4 representing the locations Thrissur, Eranakulam and Mancombu (Alappuzha), from where they were isolated.
- The isolates were screened for their temperature tolerance and EKM2 with highest mycelial weight (6.221g) was identified to be temperature tolerant and M4 which recordedlowest mycelial weight (0.328 g) was proved to be susceptible isolate.
- From the drought screening experiment it is identified that isolate CKD which has shown higher biomass (2.172 g) is drought tolerant isolate and isolate M4 with lowest mycelia weight (1.336 g) was identified as susceptible.
- From the temperature inducing experiment it is clear that EKM2 has recorded highest weight of mycelia (12.75g) and M4 has recorded the lowest mycelia weight (4.46g) at highest temperature. Hence EKM2 was identified as superior and tolerant isolate to thermal stress and M4 as susceptible.

- EKM2 has recorded the highest number of colonies (11 cfu/ml) and M4 recorded the lowest number of colonies (3 cfu/ml) and hence EKM2 was proved as thermo tolerant isolate and isolate M4 has been identified as susceptible.
- EKM 2 has recorded the highest number of spores/ ml (0.783 X 10^{7}/ml) of PD broth at the highest temperature and lowest was observed in the isolate M4 (0.217 X 10^{7}/ml). So EKM2 has been identified as the thermo-tolerant isolate and M4 as the susceptible one.
- At all temperature levels, EKM2 has recorded the highest spore length (3.47 µm) and a smaller spore length was observed in the isolate M4 (1.63 µm).At highest temperature level isolate M4 has shown low mycelial thickness (2.65 µm) and EKM2 has shown higher mycelial thickness (3.07 µm).
- The isolates EKM2 was identified as superior to other isolates, CKD and M4.
- From the drought induction experiment it is found that, isolate CKD has shown a higher mycelial weight (3.365 g) and isolate M4 recorded lowest mycelial weight. Hence, isolate CKD has proved to be superior and M4 has been identified as the drought susceptible isolate.
- From the results, it is evident that a higher CFU/ml (4 X 10^{3}colonies/ ml) was observed in the isolate CKD and hence, CKD was identified to be tolerant to drought and M4 is found as susceptible.
- Isolate CKD has recorded a higher spore count (0.323X 10^{7} /ml) in PD broth at all PEG concentrations and M4 was the one with the lowest spore count so, CKD was proven to be a drought-tolerant isolate and M4 was found as the susceptible isolate.
- Isolate CKD was the one that has shown a higher spore length (3.15 µm) at almost all levels of PEG and was identified as the drought-tolerant isolate and isolate M4 with the lowest spore size was proved to be susceptible.
- Isolate CKD has recorded a higher mycelial thickness at all temperature levels and M 4 has shown low mycelial thickness compared. Hence, it is clear that isolate CKD is superior to other isolates EKM2 and M4.
- A higher per cent mortality at all concentrations of spores was shown by EKM2 followed by CKD. $10^{8}$and 10^{9} spores/ml dosages have shown a higher mortality rate followed by 10^{7}, $10^{6}$and 10^{5} spores/ml, were the mortality was very low compared to higher doses.

- EKM2 has shown cent per cent mortality at 10^8 and 10^9 spores/ml of dosage on the 9th day of treatment. Hence, EKM2 has proven to be a superior isolate when compared to CKD. Isolate CKD has also shown a higher mortality rate compared to M4.
- At the highest dose of 1x 10^9 spores/ ml, isolate CKD has recorded with shorter LT_{50} (5.099 days)value and isolate M4 has recorded the longest LT_{50} (6.124 days) values at all the spore concentrations. Hence, EKM2 and CKD are proved to be elite and virulent isolates and M4 is found to be less virulent.
- The thermo-tolerant isolate (41 ^oC), EKM2 has recorded a total protein content of 0.354 mg/ml which is higher when compared to the total protein content of control (0.215 mg/ml). The drought-tolerant isolate (CKD) recorded total protein content of 0.373 mg/ml and control has shown a protein content of 0.259 mg/ml.
- The thermo-tolerant isolate EKM2 has shown a trehalose content of 1.954 mg/min/g tissue weight which was significantly higher when compared to the trehalose content of the control (1.074 mg/min/g tissue weight). The trehalose content in the drought-tolerant isolate CKD was 1.970 mg/min/g tissue weight and 1.224 mg/min/g tissue weight for control.
- The catalase activity ranged between 0.237 EU/min/mg proteins in the control to 0.386 EU/min/mg protein for the thermo-tolerant isolate. The drought tolerant isolate, CKD recorded catalase activity of 0.384 EU/min/mg protein and control showed a reduced activity of 0.240 EU/min/mg protein. The results are showing that the catalase activity of the tolerant isolates are significantly higher compared to the control.
- The thermo-tolerant isolate has shown a peroxidase activity of 0.0230 EU/min/g tissue weight which is higher compared to the peroxidase activity of the control (0.0120 EU/min/g tissue weight). The peroxidase activity of drought tolerant isolate CKD was recorded as 0.0236 EU/min/g tissue weight and that of its control was 0.0130 EU/min/g tissue weight.
- The thermo-tolerant isolate CKD recorded higherprotease activity of 0.490 EU/min/mg protein compared to the control (0.467 EU/min/mg protein). The drought -tolerant isolate has shown a protease activity of 0.621 EU/min/mg protein which is significantly higher compared to that of its control (0.483 EU/min/mg protein).

- At 25 min of reaction, the thermo-tolerant isolate has shown a higher lipase activity (10.000 µmol fatty acid/ml) than the control (8.667 µmol fatty acid/ml).At 25 min of the reaction the drought tolerant isolate has shown a lipase activity of 11.00 µmol fatty acid/ml which is higher compared to that of its control (5.667 µmol fatty acid/ml).
- On the 5^{th} day, the thermo-tolerant EKM2 has shown a higher chitinase activity (1.140 µg of N- acetyl glucose amine/min/ml of broth) followed by the control with 0.548 µg of N- acetyl glucose amine/min/ml of broth. The highest chitinase activity of the drought-tolerant CKD reported on the 5^{th} day was 3.062 µg of N- acetyl glucose amine/min/ml of broth and that of control was 0.074 µg of N- acetyl glucose amine/min/ml of broth.
- Low amount of protein content was detected in the isolates grown in stressed conditions and heat shock protein bands were not detected in the gel in SDS-PAGE analysis after silver staining.
- The temperature induction had up regulated heat shock proteins of range 25 kDa to 35 kDa and expressed heat shock proteins in the range 100 kDa and 180 kDa molecular weight in the isolate EKM2 confirming its heat tolerance.
- Drought induction on the other hand did not showed the presence of any heat shock protein in CKD when compared to the control. Both CKD and control had shown similar protein profiles and proved that drought did not cause expression of heat shock proteins.

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ABSTRACT

NOVEL STRAINS OF *Metarhizium anisopliae* SOROKIN. (ASCOMYCOTA: SORDARIOMYCETES) WITH ENHANCED ABIOTIC STRESS TOLERANCE

by

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ABSTRACT OF THESIS

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Abstract

The green muscardine fungus, *Metarhizium anisopliae* Sorokin is widely used in the management of various crop pests. However the efficacy of *M. anisopliae* in the field is often inconsistent, mainly due to the environmental stresses such as temperature extremes, drought, UV radiation, *etc*. To circumvent these hurdles, the present study was undertaken to develop novel strains of *M. anisopliae* with enhanced environmental stress tolerance.

The *Metarhizium* isolates, EKM2, CKD, and M4 were collected from the repository maintained at the Department of Agricultural Entomology and were screened for their temperature and drought tolerance. The isolate EKM2 recorded the highest mycelial weight (6.221g), while the isolate M4 recorded the least mycelial weight (0.328 g) and the least sporulation at the highest temperature of 38^0C. The screening for drought tolerance was conducted at various levels of Polyethylene glycol (PEG) concentrations in which the isolate CKD has shown higher biomass (2.172 g) with higher sporulation, while isolate M4 recorded the lowest mycelial weight (1.336 g) at the maximum PEG concentration of 39 per cent. The three selected isolates of *M. anisopliae* (CKD, EKM2 and M4) were subjected to further experiments.

The *Metarhizium* isolates (CKD, EKM2 and M4) selected from the preliminary screening were continuously subjected to temperature stress treatments starting from 35^{o}C to 42^{o}C. At the highest temperature of 41^{o}C, the isolate, EKM2 recorded the highest mycelial weight (1.336 g), number of colonies (18.66710^3 cfu/ml), number of spores (0.783 X 10^{7}/ml), spore size (3.470 µm) and mycelial thickness (3.07 µm). At the same temperature, the isolate, M4 recorded the lowest mycelial weight (0.63g), number of colonies (3 cfu/ml), number of spores (0.217 X 10^{7}/ml), spore size (1.637 µm), and mycelial thickness (2.65 µm). Hence EKM2 has been identified as the thermo-tolerant isolate and M4 as the susceptible one. The thermo-tolerant isolate EKM2 was grown continuously for 6 successive generations at the same temperature level of 41^{o}C in order to stabilize the stress tolerance.

The *Metarhizium* isolates (CKD, EKM2, and M4) selected from the preliminary drought screening experiment were also continuously subjected to drought-inducing treatments at PEG concentrations starting from 25 to 40 per cent. At the highest level of PEG concentration (40 %), the isolate CKD showed higher mycelial weight (3.365 g), spore count (0.323X 10^7 /ml)), number of colonies (4 X 10^3colonies/ ml), spore size (3.15 µm), and mycelial thickness (2.723µm). The isolate, M4 recorded the lowest mycelial weight (0.808g), number of colonies (2 cfu/ml), spore count (0.243 spores/ml), spore size (4.493 µm), and mycelial thickness (2.543 µm) at the same PEG concentration. Hence isolate CKD is identified as drought tolerant and M4 as susceptible. The drought-tolerant isolate, CKD was grown continuously for 6 successive generations at the same PEG concentration level of 40 per cent in order to stabilize the stress tolerance.

The selected tolerant isolates were evaluated against wax moth larva (*Galleria mellonella*) to identify their biocontrol efficacy. Isolate EKM2 has shown cent per cent mortality at the dosage of 10^8 and 10^9 spores/ml on the 9^{th} day after treatment. At the highest dosage of 1x 10^9 spores/ ml, the isolate CKD recorded a lower LT_{50} (5.099 days) value, and isolate M4 recorded the highest LT_{50} (6.124 days) value. The isolates EKM2 and CKD were found to be more virulent while M4 was less virulent.

The thermo-tolerant isolate, EKM2 recorded a total protein content of 0.354 mg/ml, and the drought-tolerant isolate (CKD) recorded a total protein content of 0.373 mg/ml. The thermo-tolerant isolate EKM2 has shown a trehalose content of 1.954 mg/min/g tissue weight which was significantly higher compared to the control (1.074 mg/min/g tissue weight). The trehalose content in the drought-tolerant isolate CKD (1.970 mg/min/g) was also higher when compared to the control (1.224 mg/min/g). The catalase activity ranged between 0.237 EU/min/mg proteins in the control to 0.386 EU/min/mg protein in the thermo-tolerant isolate EKM2, while the catalase activity of the drought-tolerant isolate, CKD was 0.384 c protein which was higher when compared to its control (0.240 EU/min/mg protein). The thermo-tolerant isolate recorded a peroxidase activity of 0.0230 EU/min/g tissue and that of drought-tolerant isolate CKD was 0.0236 EU/min/g tissue weight. The protease activity of thermo-tolerant isolate CKD was 0.490 EU/min/mg protein and that of the drought-tolerant isolate was 0.621 EU/min/mg protein.

At 25 minutes of reaction, the thermo-tolerant and drought-tolerant isolates have shown higher lipase activity (10.000 µmol fatty acid/ml and 11.00 µmol fatty acid/ml respectively) when compared to their respective controls. On the 5^{th} day, both the thermo-tolerant and drought- tolerant isolates recorded significantly higher chitinase activity (1.140 µg of N- acetyl glucose amine/min/ml of broth and 3.062 µg of N- acetyl glucose amine/min/ml of broth respectively) than their controls.

The temperature induction had up-regulated heat shock proteins of 25 kDa and 35 kDa in heat-induced isolate EKM2. The isolate also expressed heat shock proteins in the range of 100 kDa molecular weight. Drought tolerant isolate (CKD) on the other hand did not show the presence of any heat shock protein when compared to the control.

The study could develop isolates of *M. anisopliae* which are tolerant to temperature and drought stress, with high virulence as well as higher biochemical activity.