# EVALUATION OF ENTOMOPATHOGENIC FUNGI ISOLATE(S) FOR MANAGEMENT OF MELON FRUIT FLY (*Zeugodacus cucurbitae)* (Coquillette)

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## DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

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## DEDICATION

I dedicate this thesis to my parents Lameck Onsongo and Truphena Onsongo for their support, prayers and encouragement during my study. I also dedicate this thesis to my nephew Tukuza, niece Zawadi, my sisters, brother, all relatives and friends for their prayers and continued moral support.

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| AFA | Agricultural Food Authority |
| AFFP | African Fruit Fly Programme |
| AGRA | Alliance for a Green Revolution in Africa |
| ANOVA | Analysis of Variance |
| APU | Arthropod Pathology Unit |
| CRD | Completely randomized design |
| D12 | 12 hours of darkness |
| DRIP | Dissertation Research Internship Programme |
| EPF | Entomopathogenic Fungus |
| EU | European Union |
| FL | Fiducial Limit |
| GDP | Gross Domestic Product |
| *icipe* | International Centre for Insect Physiology and Ecology |
| IPM | Integrated Pest Management |
| KES | Kenyan Shillings |
| KHCP | Kenya Horticulture Competitiveness Project |
| L12 | 12 Hours of Light |
| LAS | Leica Application Suite |
| LC | Lethal Concentration |
| $LT_{50}$ | Lethal Time (time taken to kill 50% of tested population) |
| $LT_{90}$ | Lethal Time (time taken to kill 90% of tested population) |
| MRL | Maximum Residue Levels |
| n.d. | Not Dated |
| R | Software for Data Analysis |
| RCBD | Randomized Complete Block Design |

| | |
|---|---|
| RH | Relative Humidity |
| SDA | Sabouraud Dextrose Agar |
| SIT | Sterile Insect Technique |
| *Spp* | Species |
| UK | United Kingdom |
| UV | Ultra Violet |

## ABSTRACT

The melon fly, *Zeugodacus cucurbitae* (Coquillett), is a serious pest of cucurbits, tree fruits and related crops in most continents of the world, including Africa. To manage the pest, most farmers in sub-Saharan Africa rely on synthetic chemicals which have detrimental effects to the environment and the habitat. Excessive use of chemicals may also lead to development of pest resistance and *Z. cucurbitae* has reportedly shown the most increased resistance to the used insecticides. For sustainable cucurbit production, alternative methods for the management of fruit flies, that are economically sustainable and environmentally friendly, are recommended to reduce overreliance on chemical usage. The objective of this study was to identify and evaluate the best fungal isolate for management of *Z. cucurbitae* in cucurbits. Pathogenicity of thirteen isolates of *Metarhizium anisopliae* and two of *Beauveria bassiana* against *Zeugodacus cucurbitae* adults was evaluated in the laboratory. Adult fruit flies were exposed to 0.3g of dry conidia, evenly spread on a contamination device and mortality monitored daily. All isolates tested were pathogenic to the adult melon fly and mortality ranged between 20 - 94% at 5 days' post treatment. The most pathogenic isolates were ICIPE 18, 30 and 69 causing the highest mortality of 87%, 81% and 94% respectively at 5 days' post treatment. The $LT_{50}$ values of the most pathogenic isolates ranged between 4–5 days. Two of the most promising isolates were tested on pupa emergence by spraying suspension of different concentration of the selected isolates on soil before introduction of mature (last instar) larvae. The fungi was able to suppress emergence to a range of 3 to 52%. The concentration of $1 \times 10^8$ ml/$10^7$ of both ICIPE 18 and 69 isolates was the most effective causing mortality of 53% and 74% respectively. The three selected isolates, were then assessed against *Z. cucurbitae* adults at temperature regimes of 15, 20, 25 and 30°C. The optimum temperature for all isolates was found to be 25 and 30°C. Mortality from the three isolates ranged between 28.8% and 90% across the different temperatures. The isolates ICIPE 69 and ICIPE 18 recorded the highest mortality of 82% and 90% and the shortest $LT_{50}$ values of 2.61 and 2.63 days, respectively, at 30°C. However, ICIPE 69 had the highest conidia production of $90.5 \times 10^7$ at 30°C and was therefore selected for global mapping to predict its efficacy against *Z. cucurbitae* using the geospatial temperature data layer and the best fitted quadratic model. The map showed that the isolate would be highly effective in the tropics than in temperate climates. Laboratory tests confirmed compatibility of the selected ICIPE 69 isolate with Cue-lure, a commercially available attractant on adult melon fly. In addition, laboratory bioassay to test the potential for horizontal transmission showed that males and females exposed to *M. anisopliae* conidia (donors) became infected and exhibited 100% mortality. The recipients resulted to male and female mortality of 97% and 86% respectively, after 10 days of exposure, thus confirming the ability of fruit flies to transmit inoculum to other flies. It was also shown that the fungus affects the number of eggs oviposited, although the hatchability is not affected. This therefore identifies ICIPE 69 as a potential isolate for the management of melon fly with effective results at optimum temperatures and can be used together with the Cue-lure in the field set up.

# CHAPTER ONE
# INTRODUCTION

## 1.1 Background Information

About 70% of people living in Sub–Saharan Africa practice agriculture on small scale and this provide around 80% of their food supply (AGRA, 2014). Agricultural sector contributes to about 30% of the GDP and is a source of both direct and indirect employment in Kenya (Diao *et al*., 2010). Among different sectors in Kenya, horticulture sector has grown to become the second largest foreign exchange earner. The sector is also a major contributor to food needs in the country as well as direct and indirect employment (Badii *et al*., 2015). Kenya's ideal tropical and temperate climatic condition makes it favorable for production of wide range of fruits, vegetables and flowers (AFA, 2016). However, the industry faces significant challenges, including poor production and market systems, rising cost of inputs such as fertilizers and agrochemicals, lack of technical knowhow, germplasm conservation and the changing biotic and abiotic environmental factors (Tshilidzi *et al*., 2016).

Globally, cucurbits are widely cultivated and consumed and they are a good source of several vitamins and minerals (USAID, 2014). They are ranked among the major vegetable fruits grown in Kenya and exported abroad for their nutritional value and economic significance as foreign exchange earners (Tshilidzi *et al*., 2016). The major species of cucurbits grown in Kenya are butternut (*Cucurbita moschata*), pumpkin (*Cucurbita maxima*), cucumber (*Cucumis sativus*), courgettes (*Cucurbita pepo*), and watermelon (*Citrullus lanatus*) (AFA, 2016). Production of cucurbits is however, faced by various constraints, key among them is the infestation by tephritid fruit flies (Shafiq *et al.*, 2012). The fruit flies are known to cause huge economic losses not only to cucurbits but to the horticulture industry at large (Badii *et al*., 2015).

Cucurbits are among the major hosts of fruit flies recorded in different countries and this makes them vulnerable since they attack them (Leblanc *et al*., 2012). The losses caused can be direct or indirect. Female fruit flies lay eggs under the skin of the fruits. The eggs hatch into larvae that feed in the decaying flesh of the crop and the infested fruits quickly

rot and become inedible or drop to the ground causing direct losses (Magagula and Nzima, 2013). There are also indirect losses associated with quarantine restrictions. Fruit fly infestation and sometimes the mere presence of the flies in a particular country may restrict the trade and export of fruits to markets abroad (Vargas *et al.*, 2015). The primary tephritid fruit flies that cause the highest percentage of losses in Africa have been documented to be in the genera, *Bactrocera*, *Ceratitis* and *Dacus* (Soto *et al.*, 2016). Pests from the genus *Bactrocera* are the most destructive insects because the adults have high dispersive powers, reproductive rate and are extremely polyphagous (Dhillon *et al*., 2005).

In the genus *Bactrocera*, Peach fruit fly (*Bactrocera zonata*), melon fruit fly (*Bactrocera/Zeugodacus cucurbitae*) and oriental fruit fly (*Bactrocera dorsalis*) are the three most notorious, destructive and widespread species of fruit flies in the tropics (Ahmad and Ali, 2014). There are more than 125 plant species, belonging to different families, which are considered hosts of *Z. cucurbitae* (Weems *et al*., 2018). Different studies have however shown that cucurbit hosts are most preferred over non-cucurbit hosts (Mwatawala *et al.*, 2009). *Zeugodacus cucurbitae* causes direct losses in the yield and affects marketability of fruits and in addition, poses considerable threats to the quarantine security and thus to international horticultural trade throughout the world (Shafiq *et al*., 2012).

Currently, applied integrated pest management (IPM) techniques for pest fruit flies include attract-and-kill (e.g. male annihilation technique and mass trapping) using a male lure such as Cue-lure, field sanitation, protein baits, biological control with braconid parasitoids, and sterile insect releases (Leblanc *et al.*, 2013). One of the mostly used method for the management of *Z. cucurbitae* is the use of insecticides, which may be unfriendly to the environment and to the consumers, and can lead to development of insecticide resistant pest populations (Ryckewaert *et al*., 2010). This has resulted in the rising need for searching for biological control alternatives. Various isolates of entomopathogenic fungus (*Metarhizium anisopliae*, *Beauveria bassiana* and *Isaria fumosorosea)* have shown effectiveness against some other fruit fly species e.g. *Ceratitis*

*capitata* (Khlaywi *et a*l., 2014). The study was aimed at selecting and evaluating the efficacy of entomopathogens for the control of *Z. cucurbitae.*

## 1.2 Problem Statement

Some cucurbits are generally regarded as major horticultural crops in Kenya because of the role they play in improving food and nutritional security. However, their production is highly constrained by pests, especially tephritid fruit flies which are the most notorious causing a loss of 30-100%. In Kenya, tephritid fruit flies are classified as quarantine pests and have led to the banning of some crops from being exported. *Zeugodacus cucurbitae* is among the most destructive pests in cucurbits and is ranked highly in the quarantine list and is a major threat to horticultural production in Africa. The problem is compounded by restricted use of chemical pesticides, which cause adverse effects to environment and human health and introduction of the uniform enforcement of strict quarantine and maximum residue levels (MRLs) in the horticultural markets. Excessive use of chemicals also leads to development of pest resistance and *Z. cucurbitae* has reportedly shown increased resistance to the used insecticides (Vontans et al., 2011). This underscores the need to look for alternative control methods for this destructive pest. Current use of entomopathogens in pest control offers a cost-effective and efficacious alternative to chemical insecticides. However, the effectiveness and efficacy of entomopathogens for the control of Z. cucurbitae has not been exhaustively assessed, hence this study.

## 1.3 Justification

Due to the many limitations of chemical control of tephritid fruit flies, there is need to explore alternative control methods, for management of *Z. cucurbitae.* Biological control using entomopathogenic fungus has been shown to be effective in controlling different species of tephritid fruit flies (Qazzaz *et al.,* 2015). This method is more sustainable compared to chemical control since it's safe to human beings, the environment and to non-target organisms. The method is also cheaper in the long term. Various isolates of *B. bassiana* and *M. anisopliae* have shown pathogenicity to larvae and adults of *C. capitata, C. cosyra, C. fasciventris, C. rosa and C. anonae* in the laboratory and in the field (Khlaywi *et al.,* 2014). However, there are limited studies on pathogenicity of

entomopathogenic fungus to *Z. cucurbitae*. Identification of the efficacious entomopathogens for the control of *Z. cucurbitae* will significantly reduce problems faced by not only the cucurbit farmers but also the horticultural industry at large. Following the identification of the most effective entomopathogenic fungal isolates, it was prudent to investigate their effectiveness in different thermal conditions, and their compatibility with commercially available pheromone lures. This enabled issuance of proper recommendations to the farmers whose adoption will contribute significantly to the management of *Z. cucurbitae* and reduce the cost of production and yield losses associated with the pest. This will subsequently increase farmers' income, improve their livelihoods and contribute to food and nutritional security in Kenya.

## 1.4 Research Hypotheses

1. There is no significant difference in virulence to *Z. cucurbitae* among different isolates of entomopathogenic fungus.
2. Temprature has no significant effects on efficacy of selected fungal isolates in the control of *Z. cucurbitae*.
3. There is no compatibility between selected fungal isolates and a commercial pheromone lure.
4. Horizontal transmission of fungal spores from infected to healthy flies is not effective as a management strategy for melon fruit flies.

## 1.5 Research Objectives

### 1.5.1 Broad Objective

To identify and evaluate the best fungi isolate(s) for biological management of *Z. cucurbitae* and hence contribute to the control of *Z. cucurbitae* infesting cucurbits.

### 1.5.2 Specific Objectives

1. To select entomopathogenic fungi isolate(s) with high virulence to *Z. cucurbitae*.
2. To evaluate performance of the selected isolate(s) under different temperature regimes.
3. To evaluate the compatibility of the selected isolate(s) with a commercial pheromone lure (Cue-lure).

4. To evaluate the effectiveness of horizontal transmission of fungal spores from infected to healthy flies as a management strategy of melon fruit fly.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 Cucurbits and their Production in Kenya

Cucurbits belong to the family Cucurbitaceae, which is a taxonomic unit containing essential vegetables that are important sources of vitamins, minerals and fiber (Abbey *et al*., 2017). They are among the largest group of tropical vegetables, consisting of the cucumber (*Cucumis sativus*), bitter gourd (*Momordica charantia*), bottle gourd (*Lagenaria siceraria*), pumpkin (*Cucurbita moschata*), Indian squash (*Praecitrullus fistulosus*), ivy gourd (*Coccinia indica*), muskmelon or cantaloupe (*Cucumis melo*), summer squash (*Cucurbita pepo*), winter squash (*Cucurbita maxima*), and watermelon (*Citrullus lanatus*) (Butterwick, 2016). Cucurbit production in Kenya is increasing annually probably due to the growing demand in line with the family's economic and nutritional value (AFA, 2016).

## 2.2 Pests of Cucurbits

Cucurbit farming in Kenya like elsewhere around the world is challenged by both biotic and abiotic stresses. However, tephritid fruit flies are recorded as the most notorious pests when it comes to cucurbit losses (Vargas *et al*., 2015). Cucurbits are attacked by a variety of pests and diseases. Viral diseases such as cucumber mosaic virus, bacteria diseases such as vine decline disease and bacterial wilt and fungal diseases such as powdery mildew contribute to yield losses in cucurbit farms (Abou-Jawdah *et al.*, 2000; Romero *et al.*, 2004). Major insect pests which infest cucurbits include tephritid flies, aphids, thrips and whiteflies (Vargas *et al*., 2015). Tephritid fruit flies are the most notorious pests of economic importance on cucurbits in Kenya and beyond (Ryckewaert *et al*., 2010).

## 2.3 Classification and Distribution of *Z. cucurbitae*

Tephritid fruit flies belong to the order Diptera, the Superfamily Tephritoidae, the Family Tephritidae and the Subfamily Dacinae (Kambura, 2016). The family Tephritidae is the largest family of the order diptera (Oreste *et al.*, 2015) and is categorized as one of those having the most damaging pests in the horticultural industry. Tephritid fruit flies are

distributed in the tropical, subtropical and temperate regions of the world (Badii *et al*., 2015) as shown in Appendix 1 (Geographical Distribution of Melon Fly, *Z. cucurbitae*). The genera of *Bactocera, Dacus, Ceratitis, Trirhithrum, Anastrepha* and *Rhagoletis* contain economically important species of horticultural crops worldwide (Shafiq *et al.*, 2012; Kibira *et al*., 2015). It is estimated that 180 fruit fly species are of economic importance (Qin et al., 2015). Among the *Bactrocera* species identified, *B. latifrons, B. cucurbitae, B. invadens* and *B. zonata* are the common species in Sub- Saharan Africa (Kambura, 2016). However, in Kenya the most common are *B. dorsalis*, *B. cucurbitae* and *B. latifrons* (Badii *et al*., 2015). Forty-three species have been described under the genus *Bactrocera* including cucurbitae (Foba *et al*., 2012). Amongst these, *Z. cucurbitae* (Coquillett) [formerly classified as *Bactrocera cucurbitae*] is a major threat to cucurbits (Dhillon *et al*., 2005; De Meyer *et al*., 2015). The melon fruit fly is distributed all over the world, but India is considered as its native home (Ganie *et al*., 2013).

### 2.4 Economic Importance of Fruit Flies

The direct and indirect losses to fruits and vegetables to due to Tephritid fruit flies is very high. They attack the harvestable parts and the losses cannot be compensated since the damage is irreversible (Kambura, 2016). Direct damage is when a female oviposits on the fruit (Plate 2.1A) causing punctures on the fruit which acts as an entry point of opportunistic diseases. Also the eggs oviposited hatch into larvae which then feed on fruit tissue leading to premature ripening and falling of fruits and rotting (Břízová *et al*., 2015) as shown in plate 2.1B & C. Sometimes pseudo- punctures (punctures without eggs) are possible, and this reduces the market value of the produce (Ekesi, 2010) as shown in plate 2.1D. A study carried out in Hawaii (Sapkota, 2010) showed that pumpkin and squash are subject to heavy damage since the fruit flies can oviposit even before fruit set. The eggs are laid into unopened flowers or ripe fruits and the larvae successfully develop. After some days (depending on temperature and plant species), egg hatch and the larvae bore into the pulp tissue and make the feeding galleries. In the process of finding food, the larvae move to healthy tissues, where they make more galleries and in the process introduce various pathogens which make the process of fruit decomposition faster (Gujjar *et al*., 2017). Losses on bitter gourd due to melon fly infestation can range from 41 to

89% (Jacquard *et al*., 2013). Importing countries impose quarantine measures to prevent introduction of the fruit fly into recipient countries and this restricts trade causing indirect losses (Hartig n.d.; Kibira *et al*., 2015).

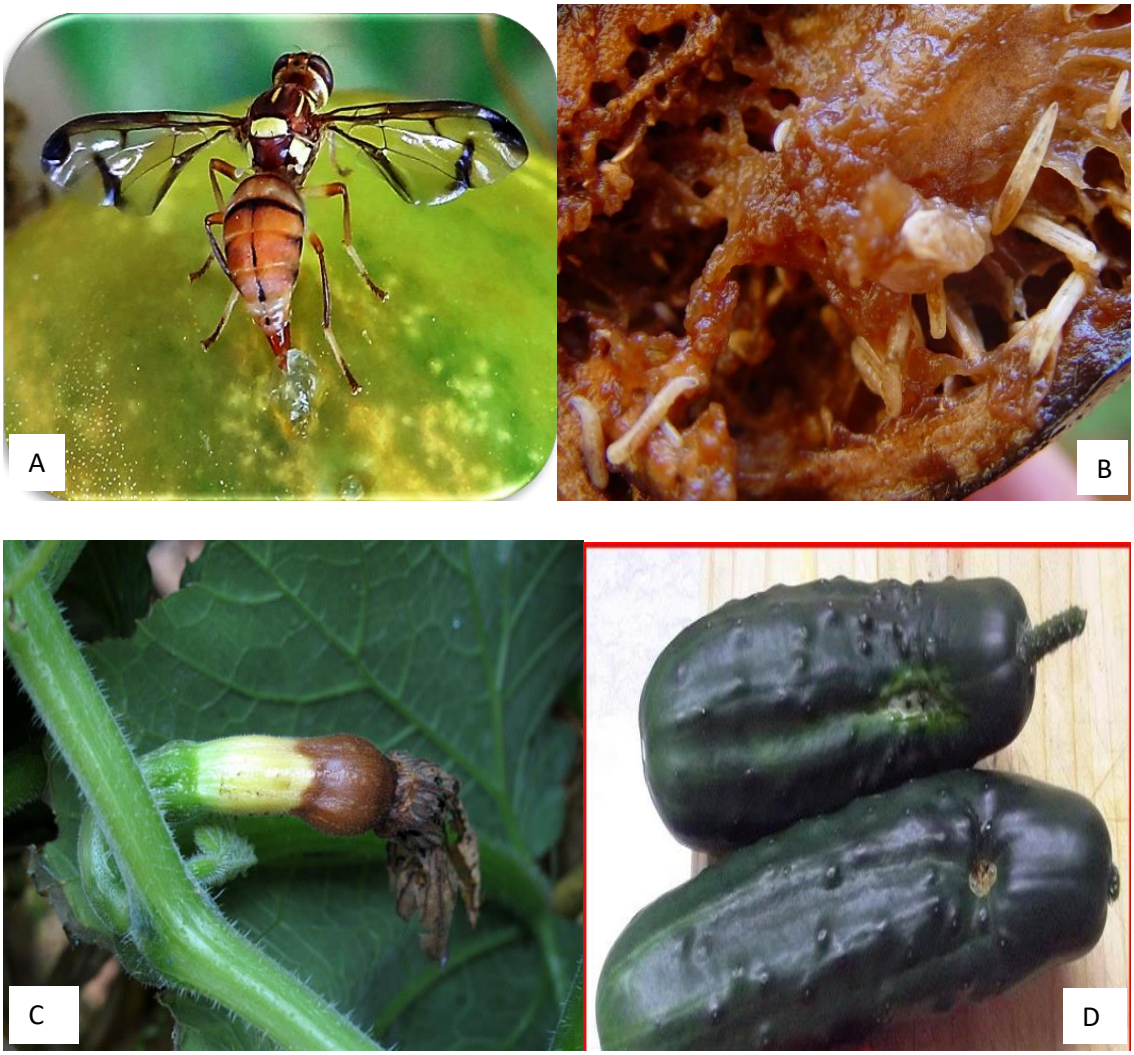


Plate 2.1: Damage caused by *Zeugodacus cucurbitae* on cucurbits.

### 2.5 Host Ranges

*Zeugodacus cucurbitae* is a polyphagous fruit fly that infests over 125 plant species mostky from Cucurbitaceae and Solanaceae families (Biasazin, 2017). However, these invasive *Bactrocera* species show preference to cucurbits in host utilization (Vayssieres *et al*., 2009). *Zeugodacus cucurbitae*, due to its polyphagous nature has been reported to be infesting 17 fruits species in West Africa (Goergen *et al*. 2011), 19 hosts out of which 11 belonged to Cucurbitaceae family in Tanzania (Mwatawala *et al*., 2009) and 11

species in Bangladesh (Leblanc *et al*., 2013). In Kenya, the mostly affected plants are butternut, watermelon, cucumber and courgettes (Kambura, 2016).

## 2.6 Biology

### 2.6.1 Life cycle

The time taken for a complete cycle of *Z. cucurbitae* in the field depends on abiotic factors such as temperature, degree of ripeness of the fruit and the moisture content among others (Dhillon *et al.,* 2005). Like other fruit fly species, melon fly's life stages are egg, larva (three instars), pupa, and adult (Dhillon *et al*., 2005). In all selected cucurbitaceous hosts, survival rates increases with increase in temperature up to the optimum temperature (Mkiga and Mwatawala, 2015). At 25°C for example, duration of development between laying and hatching was 12 to 24 h, 3.5-6.0 days for larval development and pupation and 8 to 9 days for adult emergence. The complete life span has been documented to take between 30-60 days, depending on factors like temperature (Mir *et al*., 2014).

### 2.6.2 Mating and Oviposition

Emerged flies take about 4 days to be reproductively mature (Hafraoui *et al*., 1985). In most of tephritid fruit flies, mating occurs just before dark. The mating period of between 2-4 hours was recorded and found to be sufficient for sperm transfer to occur. Fecundity (the egg laying capacity of a sexually mature adult female) is 58-92 eggs per female per day although the eggs are not laid regularly but at an intervals of 1-4 days (Mir et al., 2014). Fruit flies express differences in their preference for oviposition and host quality (Corrêa *et al*., 2018). During oviposition, the female flies uses both olfactory and visual clues to identify a suitable host (Jayanthi *et al*., 2017). Some studies have however shown that the thickness of the epicarp does not influence the oviposition (Dias *et al*., 2018). The melon fly can oviposit on already set fruit but mostly prefers unopened flowers (Courtney and Kibota, 1990).

## 2.7 Management of Tephritid Fruit Flies

Different methods have been used in the management of fruit flies. They have been grouped into: biological, cultural, chemical and legislative control methods (Deguine *et al*., 2015; Vargas *et al*., 2015; Suckling *et al*., 2016). Although some of them have been successful, it has been difficult due to their polyphagous nature. However, IPM (section 2.7.3-2.7.7) can be applied and is more efficient in the management of fruit flies (Benelli, 2015). The applicable IPM methods for control of melon fly include biological, cultural, chemical and genetic control. Biological control methods include use of entomopathagenic fungi (Wagner and Lewis, 2000), parasitoids (Mohamed *et al*., 2010), predators/natural enemies and insect traps made of sex pheromones and commercial insect attractants (Vayssieres *et al*., 2009).

### 2.7.1 Chemical Control

Pests, including fruit flies, require rigorous pest management techniques since they are attributed to high crop losses. Chemical control is the most common method used and several pesticide formulations have shown effectiveness for various fruit fly species (Hugh and Thomas, 2011). Different chemicals target various developmental stages of the fruit flies. 2-[(dimethoxyphosphorothioyl) sulfanyl]butanedioate, Diethyl (Malathion) for example, targets the adults while O,O-Diethyl O -[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate (diazinon), target the popping larvae and emerging adults (Hugh and Thomas, 2011). However, overdependence on chemicals has led to a number of detrimental effects like accumulation of pesticide residues in the environment, mortality of non-target species that can be beneficial to the ecosystem, development of resistance (Appendix 2) and also exposing the consumers and producers into health risks (Paoletti and Pimentel, 2000; Simmons and Gurr, 2005; Desneux *et al*., 2006; Adiloğlu, 2016).

### 2.7.2 Physical Control

This involves having a physical barrier between the host fruits and the egg laying female flies. Wrapping a developing fruit with a protective cover is the most common method used. This is done before the fruits reach the stage of maturity at which they are

susceptible to infestation (Sarwar, 2015). This technique is however tedious and therefore only applicable to small scale farmers. Also, this method may not be very effective as some species of fruit flies, especially those attacking cucurbits oviposit at a tender age of flowering where bagging might not be possible (Sarwar, 2015).

### 2.7.3 Cultural Control

This involves a range of crop production practices. Some of the commonly used techniques include choosing a tolerant variety, maintaining field sanitation by collecting and destroying infested, fallen, damaged and over-ripe fruit, and raking and ploughing to expose the pupae to sunlight and predators (Dias *et al*., 2018). The discarded fruits can be kept in sealed plastic bags under sun for around 10 days for most them to die. Fruit fly infestation can also be avoided by harvesting crops at a stage of maturity at which the fruit or vegetable is not susceptible to fruit fly attack (Allwood *et al*., 2001). This method, however, cannot be effective on its own but can be useful when it is used to complement other methods.

### 2.7.4 Sterile Insect Technique

Sterile Insect Technique (SIT) aims at reducing the population of a given species by introducing sterilized males to increase the chances of sterile males mating with wild females. It has been effective on controlling Mosquito-Borne Diseases (Alphey *et al*., 2010) and the Lepidoptera (Simmons *et al*. 2010). However, females that move into an area under treatment, are not affected by the presence of sterile males and can therefore oviposit in the fruits. SIT is thus only effective when applied on an area-wide basis (Hendrichs *et al*., 2006). This method also relies on rearing millions of flies for release and is species specific.

### 2.7.5 Male Annihilation Technique

This strategy uses attract and kill technique and consist of a bait (lure) which attracts the pest onto a killing agent (insecticide/ fungus). Most effective lures attract the males, which will expose them to the killing agent and therefore reduce the population of the males of the fruit flies hence reducing the chances of mating (Hafsi *et al*., 2016). Male

lures such as Cue-lure, Methyl Eugenol and Trimedlure are used. In Hawaii, Male Annihilation Technique using Amulet Cue-lure and Methyl Eugenol showed effectiveness and populations of *B. dorsalis* and *B. cucurbitae* were significantly reduced (Vargas *et al*., 2005). The study concluded that the tactic is useful if it is used with an "area-wide" suppression strategy.

### 2.7.6 Biological Control

Use of biological control is efficient in reducing the pest population and is also an eco-friendly pest management strategy, which can lead to minimal use of chemicals. Generally, the biological control involves use of natural enemies, parasitoids, predators, and pathogens (Dias *et al*., 2018), which act by feeding on the pest, parasitizing the pest and causing diseases. For example, Nematodes species *Steinernema weiseri, Steinernema feltiae, Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, isolated from Turkish soils, have been found to be effective on larvae of *C. capitata* (Karagoz *et al*., 2009), *S. feltiae* on *Bactrocera zonata* (Mahmoud, 2018) and *S. feltiae, S. carpocapsae* and *H. bacteriophora*, on *Bactrocera tryoni* (Langford *et al*., 2014).

### 2.7.7 Natural Enemies of Fruit Flies

#### 2.7.7.1 Predators

Most predators of fruit flies belong to families Coccinellidae, Staphylinidae, Formicidae, Dermaptera, Pentatomidae, Coreidae, Carabidae and Chrysopidae (Peters, 1997). Wasps, geckos, birds, ants, staphylinid beetles, toads and spiders can also prey on fruit flies (Daniel, 1976; Hendrichs and Hendrichs, 1998; Mele *et al*., 2009; Riccucci, 2014). A study by Fernandes *et al*. (2012) showed that ants, alongside other factors e.g. soil properties, can pray on *Anastrepha* larvae and reduce the population. However, some predators, e.g. birds, can also be detrimental by even preying on parasitoids hence a disadvantage.

#### 2.7.7.2 Parasitoids

Fruit flies are commonly parasitized by opiine wasps (Hymenoptera: Braconidae: Opiinae). Depending on whether the parasitoid is egg or larvae parasitoid, the wasps

oviposit their eggs into the eggs or larvae of fruit flies where the wasp larvae will develop. The egg will hatch and the larvae continues to develop until after pupation when the wasp finally kills the fly pupa and emerges as a new adult wasp (Lenteren and Roermund, 1996). Braconidae family contains most of the parasitoids of fruit flies and other families include Chalcididae, Eulophidae, Cynipidae and Pteromalidae. *Diachasmimorpha longicaudata* and *D. kraussii* have been identified as parasitoids of the olive fruit fly (Sime *et al*., 2006; González *et al*., 2007). *Fopius arisanus* (Sonan) and *D. krausii* have led to population decrease on Mediterranean fruit flies, *Ceratitis capitata* (Rendon *et al*., 2006), while *Psyttalia concolor*, a larval–pupal endoparasitoid is effective on a number of tephritid fruit flies (Canale and Benelli, 2012).

### 2.7.7.3 Effectiveness of Entomopathogenic Fungi to Control Pests

An entomopathogenic fungus is a parasite of insect which will result into killing or disabling them (Wagner and Lewis, 2000). The fungus spores act by attaching on the external body surface of insects. If the conditions are favorable, the spores will germinate, grow and colonize the insect's cuticle. Different studies have been done to check on the effectiveness of entomopathogenic fungus to control pests. A formulation of *Beauveria bassiana* was found to reduce adult emergence and also kill *Bactrocera dorsalis* up to 99% within 8-9 days (Marri *et al*., 2016). The populations of *Drosophila suzuki* (Cuthbertson and Audsley, 2016), Alder Leaf Beetle *Agelastica alni* (L.) (Sonmez *et al*., 2017), *Spoladea recurvalis* (Opisa *et al*., 2018), Mediterranean fruit fly, *C. capitate* (Khlaywi et al., 2014), Western flower thrips *Frankliniella occidentalis* (Niassy *et al*., 2012) and Adult Pea Leafminer *Liriomyza huidobrensis* (Migiro *et al*., 2010) have been reduced by entomopathogens. Also, fungi can reduce the fecundity and fertility of the adults (Dimbi *et al*., 2009).

Among microbial control agents, entomopathogenic fungi such as *M. anisopliae* and *B. bassiana* have been used in fruit fly suppression programs in Kenya (*icipe*) and have demonstrated significant levels of reductions in population of different species of fruit flies (Ekesi *et al*., 2005; Ekesi *et al*., 2007; Rtu *et al*., 2009). More recently, Real IPM Kenya Ltd., a private company in Kenya, has undertaken commercial production various bio-pesticides based on different species and isolates of fungus.

## 2.8 Factors Affecting the Efficacy of Fungi as Biological Control Agents

### 2.8.1 Environmental factors

#### 2.8.1.1 Temperature.

Temperature is one of the major factors affecting the efficacy of entomopathogenic fungi. It affects the progression of disease and the time of death (Inglis *et al*., 2001). The optimum temperature for six isolates of *M. anisopliae* infecting three species of African tephritid fruit flies is 30°C (Dimbi *et al.,* 2004), while others are 25 to 30°C (Bayissa *et al*., 2017). Although the infection and disease can occur at temperatures of 15 to 30°C, the optimum temperature for most entomopathogenic fungi is between 25 and 30°C. Above 30°C, the sporulation and vegetative growth of most isolates is inhibited but the maximum temperature for different isolates differed especially by site of origin (Inglis *et al*., 2001).

#### 2.8.1.2 Relative humidity

This is also an important environmental factor influencing the potential of entomopathogenic fungi. This is because moisture stress can limit conidia germination and vegetative growth of the fungus hence reducing the ability of the fungus to penetrate into the host (Inglis *et al*., 2001). A study by Fargues and Luz (2000) showed that daily high humidity is among the most crucial climatic constraints for *B. bassiana* to control *R. prolixus*. Although higher relative humidity (RH) is required for effective colonization, a study by James *et al.* (1998) showed that infection can occur at as low as 50% RH. Some of the techniques used to increase the RH are by using an appropriate formulation and application of irrigation water as it improves the microclimate. For example, there was a successful infection in desert locust at 20-30% RH under field condition using oil-based conidial formulation (Bateman *et al*., 1996).

#### 2.8.1.3 Solar radiation

Solar radiation affects the persistence of the fungus in the field. This is due to Ultraviolet (UV) solar radiations, in particular UV-B spectrum which causes damage on cell

constituents such as DNA, RNA, and proteins (Mpoloka, 2008). The surviving fungal conidia after exposure to UV radiation require a long period of time to recover and restore the germination process. A study by Braga *et al*. (2001) showed that the susceptibility of the conidia to UV radiation depends on physiological state of the infective conidia.

#### 2.8.1.4 Rainfall

There are few studies on how rainfall affects the persistence of fungus on insects and on foliage. This could be because it is difficult to study rainfall as a single factor due to available interaction from other factors like solar radiation, which also affect the persistence of fungus. However, rainfall has positive implications towards fungal epizootics since it can dislodge and disperse conidia from substrates (Inglis *et al.,* 2001).

### 2.8.2 Biotic Factors

#### 2.8.2.1 The Pathogen

The ability of entomopathogens to infect by producing epizootics on the host is influenced by factors such as pathogen density, host range, genetics, dispersal, latency, virulence and persistence (Cory and Ericsson, 2010; Jaronski, 2010). For the fungus to kill the host, it is presumed that a threshold number of propagules are necessary. High propagule density in the field increases the chances of an insect coming in contact with enough or adequate number of propagules that exceed the inoculum threshold (Butt and Goettel, 2000).

The ability of an entomopathogenic fungal species to remain effective for a longer time in an environment increases the probability of an insect coming into contact with propagules to cause disease. Generally, entomopathogenic fungi gain entry through penetration of the host cuticle using a combination of hydrolytic enzymes and mechanical force (Shahid *et al.,* 2012; Vega *et al.,* 2012). After penetration to the hemocoel, the host dies due to a combination of toxin, obstruction of blood circulation, nutrient depletion and invasion of organs (Inglis *et al.,* 2001; Shahid *et al*., 2012).

### 2.8.2.2 The Insect Host

Most arthropods are hosts of fungi although host spectra vary widely, depending on fungal species. Most studies have shown that *B. bassiana* and *M. anisopliae* have much wider host ranges within the Arthropoda (Inglis *et al.*, 2001). The susceptibility of the insect pest to entomopathogenic fungus is influenced by both physiological and morphological factors (Jaronski, 2010; Ortiz-Urquiza and Keyhani, 2013). Host population density and distribution, pest population growth characteristics, host behavior and population composition are among the factors Inglis *et al.*, 2001). Age, genetics, nutrition, and exposure to injuries also influence the susceptibility (Shapiro *et al*., 1999; Inglis *et al.*, 2001). It has been reported that inadequate nutrition increases the susceptibility of the pest insect to the fungus (Inglis *et al.*, 2001; Shapiro-Ilan *et al.*, 2012). Increased host density, increases contact between the infected and uninfected populations hence favours infection and also increases availability of substrate and nutrients for pathogen growth and reproduction. This increases the quantity of inoculum available in the habitat to further cause infection (Inglis *et al.*, 2001).

After the insect pests have been exposed to the fungus, some insects tend to behave differently. For example, flies and locusts elevate body temperatures to a level that is averse to the entomopathogenic fungus in the hemocoel. There is also grooming in termites and summit disease syndrome in grasshoppers (Jaronski, 2010). The cuticle of some pests possess physicochemical properties that affect the infection process either negatively or positively. For instance, the cuticular extracts from larval *Helicoverpa zea* inhibited *B. bassiana* conidial germination while those of *Nazera. viridula* lowered the conidial of *M. anisopliae* and these were attributed to the presence of the aldehyde, (E)-2-decenal (Jaronski, 2010). As a defence mechanism, a range of immune responses are initiated once the fungus reaches the hemolymph (Vega *et al.*, 2012). However, species *M. anisopliae* and *B. bassiana* have shown capability of avoiding encapsulation in the hemocoel (Bidochka *et al.*, 2001).

### 2.8.2.3 The Host Plant

When fungus is sprayed to plants, plant-mediated effects on fungal entomopathogens can affect the entry either directly or indirectly. The direct effects include plant architecture altering spore persistence, leaf topology and surface chemistry influencing the rate of spore acquisition by the host insect and plant exudates affecting the conidia directly (Cory and Ericsson, 2010). Other direct effects include leaf modifications of microclimate affecting spore germination and herbivore-induced plant volatiles affecting sporulation or germination (Ignoffo, 1992). Potential indirect effects include changes in insect growth rate, which might alter the exposure of the insect to fungal entomopathogens and nutritional quality altering insect morphology (e.g. cuticle depth) which would influence the infection process (Cory and Ericsson, 2010). Other indirect effects include plant quality (e.g allelochemicals and nutrients), altering insect condition (e.g. immunity) and hence disease resistance and plant structure altering insect behavior, and thus fungal encounter rate (Cory and Ericsson, 2010).

## 2.9 Compatibility of Entomopathogenic Fungus with Pheromones Lures

Pheromones are chemical substances released by insects that affect the behavior and physiology of members of conspecifics (Biasazin, 2017). Tephritid fruit flies have been well managed using pheromones (Deguine *et al*., 2015). Cue-lure, a commercial male attractant, decreased fruit fly infestation on sweet gourd farms by over 40% when sprayed on the crop (Leblanc *et al*., 2013). Use of bait traps, which are insect traps made of pheromones and attractants, have proved effective in the management of tephritid fruit flies (Tinzaara *et al*., 2007; Vayssieres *et al*., 2009). However, for effectiveness of the experiment setup in the field, it's important to know if the fungus is compatible with the pheromone lure. The compatibility of fungus with thrips attractants (David *et al*., 2016) and *M. anisopliae* isolate 69 with some agrochemicals (Niassy *et al*., 2012) showed that there was a significant difference on compatibility among lures. Also, *Calpurnia aurea* has been found to be compatible with fungus (Nana *et al*., 2012)

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Insect Source and Rearing Conditions

Adult *Z. cucurbitae* were obtained from mass rearing colony maintained at *icipe* Nairobi. They were exposed to butternuts for 24-48 hours for oviposition. To hold the infested butternut, a wire mesh was placed at 15cm of a plastic container that contained sand up to a depth of 5 cm. The infested butternuts were placed on top of the wire mesh inside the container for the eggs to hatch and the larvae to pop out and drop to the sand to pupate. The pupa were collected in Petri dishes and placed in cages measuring 15cm x 15cm x 15cm for the adult to emerge. The emerged adult flies were maintained on a sugar and yeast hydrolysate based artificial diet as described by Chang *et al.* (2004), at 45%RH, 12 hours of light and 12 hours darkness (L12: D12).

## 3.2 Fungi Sources and Maintenance of Fungal Cultures

The fungal species *M. anisopliae* and *B. bassiana* used in this study were obtained from *icipe* Microbial Germplasm in Nairobi. The fungi were cultured and maintained on Sabouraud Dextrose Agar (SDA) media in Petri dishes (Plate 3.1) and incubated at ambient temperatures for 21 days in the laboratory before being used for the studies. The conidial viability test was carried out by scrapping the surface of the 21-day old fungal culture and suspending the inoculum in 15 mL of sterile 0.01% Triton in universal bottle containing glass beads that are 3 mm diameter to obtain a stock solution (Mar and Lumyong, 2012). A homogeneous suspension of conidia was then obtained by vortexing for 3 minutes and a final concentration prepared by diluting from the stock and quantifying. A volume of 0.1 mL of conidial suspension was then spread onto clean SDA plates. Each isolate was cultured on 3 plates to act as replication in a completely randomized design (CRD) and tested for viability. The plates were incubated at 26°C for a period of 16-18 hours which was followed by fixing with lacto-phenol cotton blue to terminate fungal growth. Sterile slide cover slips were placed on each plate and viability observations recorded from each plate. Viability was determined by counting a total

number of 100 conidia for both germinated and non-germinated propagules in all cultured plates for each isolate and mean percentage germination determined (Migiro *et al*., 2010).

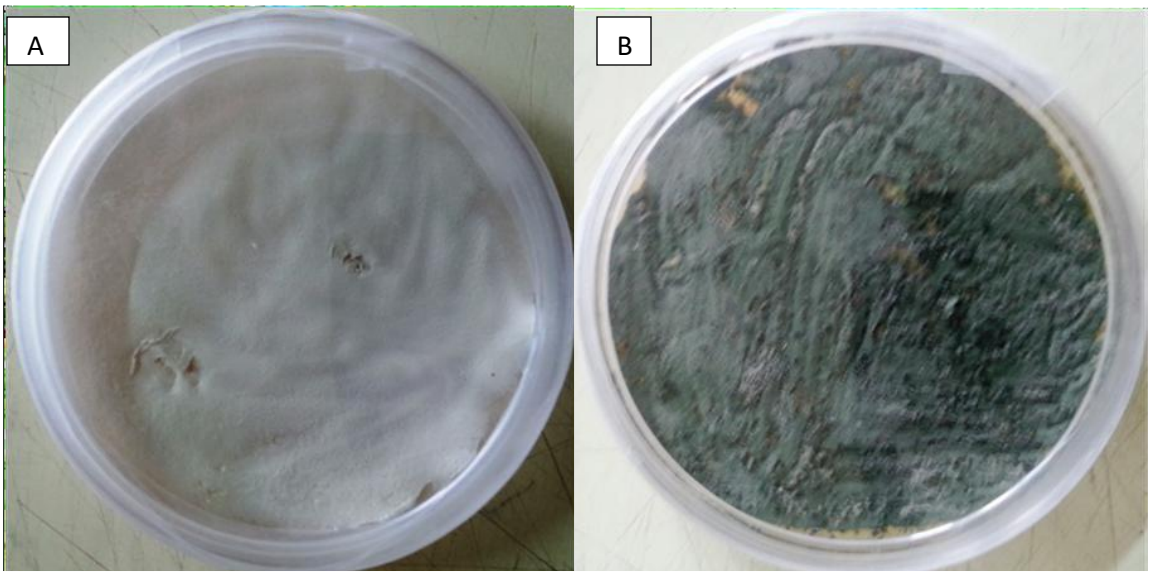


Plate 3.1: Sporulating cultures of entomopathogenic fungi used in the screening experiment. A = *Beauveria bassiana*; B = *Metarhizium anisopliae*

### 3.3 Virulence of Entomopathogenic Fungal Isolates to *Z. cucurbitae*

Thirteen (13) isolates of *M. anisopliae* and and two (2) isolates of *B. bassiana* (Table 3.1) were screened for virulence against *Z. cucurbitae*. The fungal isolates were evaluated on adult *Z. cucurbitae* in the laboratory, to determine level of mortality over time. While under laminar flow, conidia was scrapped from 21 days old culture using wire loop. A mass of 0.3 g of conidia was weighed and a spatula used to evenly spread the conidia onto a clean contaminating device. Twenty flies aged between 7-10 days were picked from the insect colony and placed in the contaminating device for 5 minutes (Qazzaz *et al*., 2015). The inoculated *Z. cucurbitae* were then transferred to cages measuring 15cm x 15cm x 15cm which was supplied with water in falcon-tube lids filled with pumice granules and artificial adult food in a Petri dish. Mortality rate, measured as a percentage of dead flies within a treatment, was observed and recorded daily until all the insects in the treatment died (Toledo *et al*., 2007). Only mycosed flies (Plate 3.2) were subjected to analysis. The experiment was laid out in a CRD with five replicates. Mycosis was recorded from the cadaver between day 2 and 5 of incubation by observing any growth of fungus on the surface using a microscope. Mortality data was corrected for natural

mortality using Abbott's formula (Abbott 1925) then normalized by arcsine transformation.

Table 3.1: Details of Fungal Isolates Tested against *Z. cucurbitae* Adults

| **Fungal Species** | **Fungal Isolate** | **Source** | **Place of Origin (Site)** | **Year of Isolation** |
|---|---|---|---|---|
| *M. anisopliae* | Icipe 07 | *Rhipicephalus appendiculatus* | Rusinga Island (Kenya) | 1996 |
| | Icipe 18 | Soil | Mbita (Kenya) | 1989 |
| | Icipe 20 | Soil | Migori (Kenya) | 1989 |
| | Icipe 30 | *Busseola fusca* | Kendubay (Kenya) | 1989 |
| | Icipe 315 | *Tetranychus uticae* | Kerugoya (Kenya) | 2006 |
| | Icipe 690 | Lepidoptera Larvae | Kenya | 2010 |
| | Icipe 62 | Soil | Matete (D.R. Congo) | 1990 |
| | Icipe 655 | Soil | Kabuti (Kenya) | 2008 |
| | Icipe 656 | Soil | Kapiti (Kenya) | 2008 |
| | Icipe 674 | Soil | Mariakani (Kenya) | 2008 |
| | Icipe 69 | Soil | Matete (D.R. Congo) | 1990 |
| | Icipe 78 | *T. nigroplagiata* | Ungoe (Kenya) | 1990 |
| | Icipe 81 | *K. angulifera* | Kaffrine (Senegal) | 2003 |
| *B. bassiana* | Icipe 279 | Coleopteran larvae | Kericho (Kenya) | 2005 |
| | Icipe 603 | Hymenoptera | Taita (Kenya) | 2007 |

Plate 3.2: A mycosed adult melon fly (*Z. cucurbitae)* due to *M anisopliae.*

### 3.3.1 Soil Inoculaton with *M. anisopliae* against *Z. cucurbitae* Pupa Emergency

A clean butternut was placed inside a cage containing adult flies for infestation. After 12hrs, the butternut was removed for the eggs to hatch and the larvae feed till the desirable stage was obtained. The larvae were collected from laboratory-infested butter nuts and placed into a sterile petri dishes. Spore suspensions of 1 x $10^6$, 1 x $10^7$ and 1 x $10^8$ conidia/ml were made from the selected fungal isolate in 0.1 % Tween 100. A volume of 20ml suspension was then sprayed on 100 g of sterile soil which was already placed in cages measuring15cm x 15cm x 15cm. A hand sprayer was used to spray the fungus and ensured that a large area of the soil came in contact with the fungus. This is mainly because limited contact with the biopesticide in treated media can inhibit the effectiveness of the fungus. Each cage was introduced with 50 larvae. Control cages were sprayed with a solution of 0.1 % Tween 100. The larvae were monitored daily until they pupated and emerged. The emerged adults were counted and immediately moved into different cages and provided with artificial diet and a source of water. Mortality was recorded daily and the cadever were removed from the cages and placed on moist filter paper in sterile petri dishes which were then parafilmed. The plates were kept at room temperature and monitored daily for mycosis. Each treatment was replicated four times.

### 3.4 Performance of the Selected Isolate(s) at different temperatures

Conidial germination, sporulation, mycelium growth rate and fungal virulence were used to establish the performance of the three *M. anisopliae* isolates previously selected under section 3.3 of this thesis. They were evaluated under temperatures of 15, 20, 25 and 30°C under continuous light conditions in a CRD with four replicates.

#### 3.4.1 Effects of Temperature on Conidial Germination

The conidial germination of the selected isolates was evaluated by harvesting the conidia of the fungus from 21-day-old cultures and placed in 3 mL of distilled water. The conidial concentrations were set at 1 x $10^7$ for each isolate from which 100 µL of the concentration was spread on 5 SDA medium plates and incubated at different temperatures under continuous light.

#### 3.4.2 Effects of Temperature on Sporulation

For sporulation, five Petri dishes of the medium were inoculated with mycelial discs (5 mm in diameter) of the selected isolates taken from 3-day-old cultures. Plates were incubated at different temperatures. After 12 days, spores from each plate were harvested and vortexed in 10 mL of triton water. The experiment was replicated four times. Spores produced per unit area were determined using haemocytometer.

#### 3.4.3 Effects of Temperature on Radial Growth

To assess the effect of temperature on radial growth, 0.1 ml spore suspension of each isolate titrated to 3 x $10^6$ conidia $ml^{-1}$ was evenly spread on SDA and allowed to grow for 3 days to obtain mycelial mats. Plugs (ca. 5 mm) of mycelium were cut from the plates using an 8-mm-diameter cork-borer and placed upside down at the center of a 90-mm Petri dish containing sterile SDA. The plates were sealed with Parafilm membrane and incubated for 12 days in the temperatures described under section 3.4 of this thesis. The radial growth was measured daily using two cardinal diameters drawn at the bottom of each plate as shown in plate 3.3.The experiment had 4 replications.

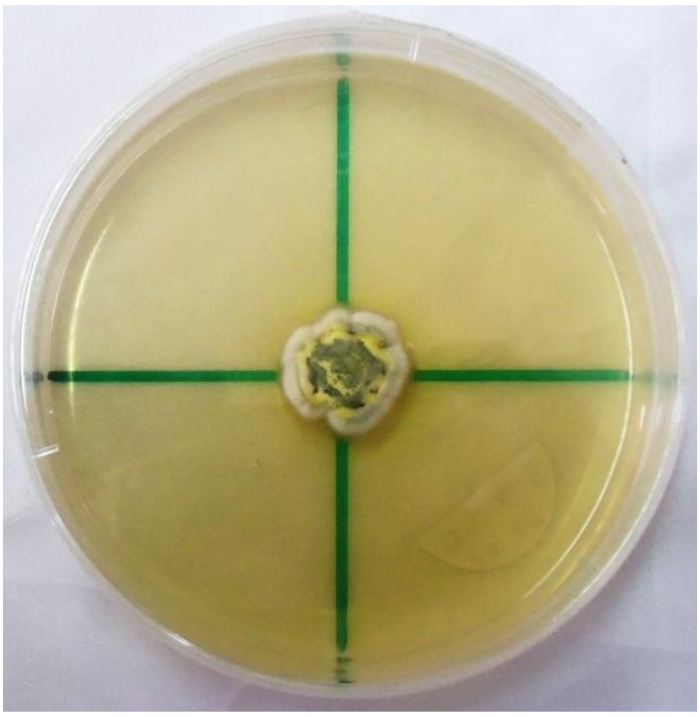

Plate 3.3: Radial growth of *M. anisopliae* on Saboraud dextrose agar (SDA) medium

### 3.4.4 Effects of Temperature on Fungal Virulence

While under laminar flow, conidia were scrapped from 21 days old culture using wire loop. A mass of 0.3 g of conidia was weighed and a spatula used to evenly spread the conidia onto a clean contaminating device (A plastic vial with a velvet material covering on the inside). Twenty adult flies (5-7 days old) were picked from the insect colony and placed in the contaminating device for 5 minutes to walk and pick fungus (Qazzaz et al., 2015). Control insects were exposed to fungus-free velvet before being transferred to ventilated Plexiglas cages. The inoculated *Z. cucurbitae* were then transferred to cages measuring 15cm x 15cm x 15cm, supplied with a cotton bud soaked in water and artificial adult food in a Petri dish. The infected flies were then incubated at 15, 20, 25, and 30°C, with four replicates at each temperature. Mortality was recorded daily for 4 days. Dead insects were surface-sterilized in 70% alcohol and 3% sodium hypochlorite followed by 3 rinses in sterile distilled water and transferred to Petri dishes lined with damp sterilized filter paper to promote fungal growth on the surface of the cadaver. Mycosis was confirmed by examining the surface of the cadaver under a microscope.

### 3.5 Compatibility of the Selected Isolates with a Commercial Lure (Cue-lure)

Conidial germination and germ tube length were used to establish the compatibility of the selected isolates with the available commercial lure. A conidia concentration of $1\times10^7$ was prepared from the stock solution of the selected isolates, after which 10 mL of the suspension was poured through a filter holder unit under aspirator vacuum and the spores were retained on a nitrocellulose filter membrane. The nitrocellulose filter membranes were then placed under a laminar flow cabinet for 30 minutes to dry and then transferred to glass desiccators for exposure to the lure (Niassy *et al*., 2012). Fungus-treated nitrocellulose membranes were exposed to the pheromone lure and sampled for viability at day 1, 2, 3, 6 and 8. The treatments were set at different temperatures of 18°C, RT (25±2°C), and 30°C to test if temperature can affect the emission of compounds. A control without the lure was also included (Nana *et al*., 2012). They were evaluated under different temperatures under continuous light conditions in a CRD with 4 replicates.

For conidial germination to be determined, the nitrocellulose filter membranes used in the experiment were removed from the desiccators and transferred into 10 mL sterile distilled water containing 0.05% Triton X-100 and vortexed to dislodge conidia. A volume of 0.1 mL of $3 \times 10^6$ conidia mL suspension was spread on SDA plates. Plates were then incubated at 26 ± 2°C, L12: D12 photoperiod and examined after 16-18 hrs for conidial germination and germ tube length. Percentage germination was then determined by counting 100 spores per plate. The length of germ tubes was measured using a Leica Application Suite (LAS EZ V1.5.0).

### 3.6 Horizontal Transmission of the Selected Isolates among *Z. cucurbitae* Adults

Before the bioassay, the flies were separated by sex (females have a sharp ovipositor at the abdomen while the males do not have).In order to inoculate the adult flies, 0.3 g of dry conidia was spread evenly into the contamination device. The device was a plastic vial with a velvet material covering on the inside, and with the bottom removed and replaced by white netting measuring 9.5 x 4.8 cm. For conidial uptake and retention**,** five groups of twenty adult flies were allowed to walk on the velvet material for three minutes after which they were transferred to respective cages measuring 15cm x 15cm x 15cm.

Ten (10) flies were taken randomly for spore uptake and retention count at 0, 2, 4, 6, 8 and 24hrs. The amount of conidia was quantified by individually transferring the insect into plastic vial containing one mL of water containing 0.05% Triton X-100. The vials were then vortexed for three minutes so as to dislodge the conidia from the body of the insect. The number of conidia obtained were estimated using a hemocytometer under a light microscope of X40 magnification.

To evaluate the transmission of inoculum between flies, four groups of 20 ten-day-old male flies were inoculated as per the above procedure. Twenty-four hours later, the treated male flies were mixed with equal numbers of ten-day-old fungus-free females and maintained together in Plexiglas cages for 24 h to allow for contact including mating. The insects were separated by sex after mixing. Another group of 20 fungus-free males and females were also held together under similar conditions and used as controls. Twenty-four hours after exposure, the flies were separated by sex and held in separate cages for 10 days at 20 - 26°C and 40 - 70% RH and their mortality recorded. The experiment was replicated four times. In similar set of experiment, four groups of 20 ten-day-old females were contaminated with fungal conidia and then mixed with equal numbers of ten-day-old fungus-free males after 24 h. Another group of 20 fungus-free females and males were also held together under similar conditions and used as controls (Dimbi *et al*., 2013).

On reproduction potential, twenty fungus-infected female flies were put in cages together with an equal number of fungus-free male flies and held together for 5 days, while provided with food and water. Butternut epicarp (cut in sphere) was used as substrate for oviposition. The eggs were collected and counted daily under a dissection microscope. Twenty eggs were randomly picked every day from every treatment and transferred to a 90-mm Petri dish lined with damp black cloth. The plates were incubated at room temperature and the number of eggs that hatched recorded daily.

### 3.7 Data Analysis

i) To determine the virulence of Entomopathogenic fungal isolates to *Z. cucurbitae*, Data was corrected for natural mortality using Abbott's formulae (Abbott 1925) then subjected to one-way analysis of variance (ANOVA) was used to analyze the mean percentage mortality after arcsine transformation to normalize the data. The means were separated using Student Newman Keuls (SNK) test ($\alpha = 0.05$).

ii) To determine the effects of temperature on germination, sporulation, mycelial growth and fungal virulence, collected data was subjected to two-way ANOVA after arcsine transformation, where necessary. Virulence data was corrected using Abbott's formula to correct natural mortality before analysis. The means were separated using Student Newman Keuls (SNK) test ($\alpha = 0.05$). Linear regression analysis was done to compare the temperature with conidial germination, sporulation, mycelium growth rate and fungal virulence.

iii) To evaluate the compatibility of the selected isolates with a commercial lure (Cue-lure), data on conidial germination and conidial germ tube length were subjected to ANOVA and means were separated using Student Newman Keuls (SNK) test ($\alpha = 0.05$). Pearson correlation analysis was done to relate conidial germination with the germ tube length.

iv) To test for the horizontal transmission of the selected isolates among *Z. cucurbitae* adults, virulence data was corrected using Abbott's formula to correct natural mortality before analysis and then subjected to analysis of variance after arcsine transformation. On the other hand, count data on number of hatched eggs was subjected to analysis of variance after angular transformation to normalize the data. Means were separated using Student Newman Keuls (SNK) test ($\alpha = 0.05$).

# CHAPTER FOUR

# RESULTS

## 4.1 Pathogenicity of Entomopathogenic Fungal Isolates against *Z. cucurbitae* Adults

In viability tests, the conidia of *M. anisopliae* isolates were incubated for 18 ± 2h at 26 ± 2°C. The germination of conidia ranged from 87 to 97% (Table 4.1). All fungal species were pathogenic to melon fly, but mortality was significantly different among the fungal isolates (Appendix 3) (P<0.001). *Metarhizium anisopliae* isolates were more pathogenic than *B. bassiana* isolates, causing mortality ranging from 29.0 to 94.4% at 5 days' post-treatment (Table 4.1). The lethal time to 90% mortality (LT90) varied from 3.8 to 6.6 days. *Metarhizium anisopliae* isolates ICIPE 69 and 18 had the shortest LT90 values of less than 4 days while the two *B. bassiana* isolates ICIPE 279 and ICIPE 603 had the longest LT90 of over 6 days, with the lethal time required to achieve 90% mortality (LT90) varying with fungal isolates (Table 4.1). Based on conidia viability, mortality against *Z. cucurbitae* and LT90 values, *M. anisopliae* isolates ICIPE 69, ICIPE 18 and ICIPE 30 were selected for further evaluation.

Table 4.1: Conidia Germination of EPF isolates and their virulence on *Z. cucurbitae* adults at 5 days post-treatment

| Fungal Species | Isolates | %Germination ± SE | %Mortality ± SE | LT90 (days) (95% FL) |
|---|---|---|---|---|
| *M. anisopliae* | Icipe 07 | 90.32 ± 3.84ab | 60.8 ± 3.29fgh | 4.74 (4.70–4.78) |
| | Icipe 18 | 94.97 ± 0.67ab | 87.2 ± 1.77b | 3.94 (3.91–3.97) |
| | Icipe 20 | 95.28 ± 0.82ab | 76.0 ± 6.30cd | 4.40 (4.36–4.43) |
| | Icipe 30 | 97.20 ± 0.45ab | 81.0 ± 2.49bc | 4.23 (4.20–4.27) |
| | Icipe 315 | 94.21 ± 1.75ab | 57.6 ± 6.11gh | 4.77 (4.72–4.82) |
| | Icipe 690 | 94.28 ± 1.65ab | 53.8 ± 3.46h | 4.90 (4.83–4.96) |
| | Icipe 62 | 90.23 ± 0.56ab | 71.6 ± 3.78de | 4.41 (4.37–4.45) |
| | Icipe 655 | 92.17 ± 2.98ab | 58.0 ± 4.30fgh | 4.84 (4.79–4.89) |
| | Icipe 656 | 90.46 ± 1.33ab | 64.6 ± 8.03efg | 4.68 (4.64–4.73) |
| | Icipe 674 | 95.56 ± 1.18ab | 29.0 ± 2.65i | 5.63 (5.53–5.73) |
| | Icipe 69 | 97.44 ± 0.72a | 94.4 ± 2.62a | 3.79 (3.76–3.82) |
| | Icipe 78 | 95.00 ± 3.00ab | 77.4 ± 3.85cd | 4.09 (4.05–4.13) |
| | Icipe 81 | 97.82 ± 0.75a | 68.0 ± 4.56def | 4.46 (4.42–4.51) |
| *B. bassiana* | Icipe 279 | 87.25 ± 3.00b | 20.0 ± 5.26i | 6.65 (6.42–6.88) |
| | Icipe 603 | 90.43 ± 3.30ab | 22.6 ± 4.2i | 6.11 (5.94–6.27) |

**Key**: Means within a column followed by the same letter are not significantly different at $p \leq 0.05$. LT90 is the lethal time in days taken to kill 90% of the adult flies; FL is fidicial limit at 95%.

### 4.2 Emergence of *Z. cucurbitae* Pupa from *M. anisopliae* Inoculated Soil

The three tested isolates were found to be effective in reducing the *Z. cucurbitae* adult emergency at all the tested concentrations. There was no significant (P>0.05) interaction between concentration and isolate (Appendix 3). There was significant (P<0.001) effect of different *M. anisopliae* conidial concentrations on average emergence of *Z. cucurbitae* from the inoculated soil. However, the fungal isolates did not significantly (P>0.05) affect the suppression of emergence. (Table 4.2).

Table 4.2: Percent emergence of *Z. cucurbitae* adults at 8 days post-exposure to various conidial concentrations of two fungal isolates

| | Fungal isolates | | |
|---|---|---|---|
| Concentration | ICIPE 18 | ICIPE 30 | ICIPE 69 |
| **Control** | 89.50 ± 3.59a | 89.5 ± 3.59a | 89.5 ± 3.59a |
| **$1.0x10^6$** | 51.50 ± 20.02ab | 39.0 ± 9.95b | 13.0 ± 2.89b |
| **$1.0x10^7$** | 31.50 ± 18.32b | 25.0 ± 4.51b | 22.0 ± 7.12bc |
| **$1.0x10^8$** | 27.57 ± 8.53b | 10.0 ± 0.82b | 2.73 ± 0.92c |
| Concentration | $F_{3,30} = 18.87$ | P <0.001 | |
| Isolate | $F_{2,30} = 3.144$ | P>0.05 | |
| Concentration x isolate | $F_{4,30} = 0.623$ | P>0.05 | |

**Key:** Means with the same lower case letter within the column are not significantly different based on SNK test at $P \leq 0.05$.

#### 4.2.1 Mortality of *Z. cucurbitae* Emerged from Inoculated Soil

The mortality of emerged adults was found to be significantly higher on inoculated soil as compared to the ones in control treatments ((P<0.001) (Appendix 3). The three tested isolates were found to be effective on *Z. cucurbitae* adults causing fly mortality ranging from 20 to 74% depending on conidial concentration applied. However, the three fungal isolates were not significantly (P>0.05) different from each other in their virulence against emerged *Z. cucurbitae* adults. On the other hand, there was significant (P<0.001) effect of different *M. anisopliae* conidial concentrations on mortality of *Z. cucurbitae* adults emerged from the inoculated soil. The highest conidial concentration of $1.0 \times 10^8$ produced the highest mortality in for isolates ICIPE 18 and 69. Consequently, there was significant interaction (P=0.04) between the isolates and conidial concentration on mortality of the emerged *Z. cucurbitae* adults (Table 4.3).

Table 4.3: Mortality of *Z. cucurbitae* emerged adults at 8 days post-exposure to various conidial concentrations of three fungal isolates

| **Conidial Concentration** | **Fungal Isolates** | | |
|---|---|---|---|
| | **ICIPE 18** | **ICIPE 30** | **ICIPE 69** |
| **Control** | 06.68 ± 1.53a | 06.68 ± 1.53a | 06.68 ± 1.53a |
| **$1.0x10^6$** | 22.50 ± 3.33bA | 34.38 ± 6.3bA | 22.74 ± 14.95bA |
| **$1.0x10^7$** | 20.23 ± 8.17bA | 51.22 ± 6.49bB | 24.91 ± 8.76bA |
| **$1.0x10^8$** | 53.39 ± 6.07cAB | 45.00 ± 2.89bB | 74.29 ± 15.25cA |
| Concentration | $F_{3,30}$ =18.87 | P<0.001 | |
| Isolate | $F_{2,30}$ =1.104 | P=0.34 | |
| Concentration x isolate | $F_{4,30}$ =2.94 | P=0.04 | |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

### 4.3 Effect of Temperature on Conidial Germination

The conidia of the three selected isolates of *M. anisopliae* (ICIPE 69, ICIPE 18 and ICIPE 30) germinated at all the temperatures tested with mean germination rates ranging from 3 to 99% (Table 4.4). The conidial germination rates for all the three isolates were significantly (P<0.001) affected by the various temperature regimes (Appendix 4). The highest conidial germination was recorded at 25°C and 30°C which were not significantly (P>0.05) different from each other but were significantly different from the lower temperature regimes of 15°C and 20°C for all the isolates (Table 4.4). The optimal temperatures for conidial germination were observed to be between 25°C and 30°C for all the three isolates (Table 4.4). Consequently, there was no significant (P>0.05) interaction between the fungal isolates and the temperature regimes with regard to conidial germination.

Table 4.4: Effect of temperature on conidial germination of *M. anisopliae* isolates

| | Fungal isolates | | |
|---|---|---|---|
| Temperature | ICIPE 18 | ICIPE 30 | ICIPE 69 |
| 15ºC | 04.26 ± 0.35c A | 03.65 ± 0.20c AB | 02.90 ± 0.28c B |
| 20ºC | 69.83 ± 1.82b A | 71.74 ± 1.27b A | 71.75 ± 2.99b A |
| 25ºC | 98.86 ± 0.48a A | 97.64 ± 0.45a A | 98.96 ± 0.49a A |
| 30ºC | 98.00 ± 0.23a A | 97.69 ± 0.41a A | 98.56 ± 0.27a A |
| Temperature | $F_{3, 36}$ = 3084.63 | P<0.001 | |
| Isolate | $F_{2, 36}$ = 1.23 | P=0.304 | |
| Temperature X isolate | $F_{6, 36}$ = 1.57 | P=0.184 | |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

### 4.4 Effect of Temperature on Fungal Growth

Temperature was found to have a significant (P<0.001) effect on fungal growth rate of all the three isolates (Appendix 4). The growth rate was found to increase with temperature with the highest growth being recorded at 30°C and the lowest at 15°C for all the three fungal isolates. There was also significant (P<0.001) differences in fungal growth rate among the three isolates. At 15°C ICIPE 18 produced faster mycelial growth than ICIPE 30 and 69 both of which were not significantly different. At 20°C, ICIPE 18 and 69 were not significantly different but they produced significantly faster mycelial growth than ICIPE 30. All the three isolates were not significantly different in mycelial growth at 25°C and 30°C (Table 4.5). Interaction between the temperature and the fungal isolates was not significant (P>0.05) with regard to fungal growth rate.

Table 4.5: Effect of Temperature on the Daily Growth Rate of *M. anisopliae* isolates

| | Fungal Isolates | | |
|---|---|---|---|
| Temperature | ICIPE 18 | ICIPE 30 | ICIPE 69 |
| 15ºC | 1.32 ± 0.10d A | 0.18 ± 0.07d B | 1.00 ± 0.06d B |
| 20ºC | 2.48 ± 0.11c A | 1.65 ± 0.13c B | 2.44 ± 0.15c A |
| 25ºC | 3.15 ± 0.16b A | 2.85 ± 0.04b A | 3.34 ± 0.13b A |
| 30ºC | 3.88 ± 0.18a A | 3.79 ± 0.15a A | 4.08 ± 0.24a A |
| Temperature | $F_{3, 36}$ =241.712 | P<0.001 | |
| Isolate | $F_{2, 36}$ =13.267 | P<0.001 | |
| Temperature x Isolate | $F_{6, 36}$ =2.006 | P>0.05 | |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

## 4.5 Effect of Temperature on Fungal Sporulation

Sporulation occurred in the fungal isolates as shown in plate 4.1. There was significant ($P < 0.001$) interaction between isolate and temperature (Appendix 4). Spore production was significantly affected by temperature ($P < 0.001$) and isolate ($P < 0.001$). The isolate ICIPE 69 produced the highest conidia spores than the other isolates at 20°C, 25°C and 30°C.The best sporulation temperature for all the isolates was found to be at 25°C (Figure 4.1).

Plate 4.1: Fungal sporulation of *Metarhizium anisopliae* on Saboraud dextrose agar (SDA) medium at 25°C

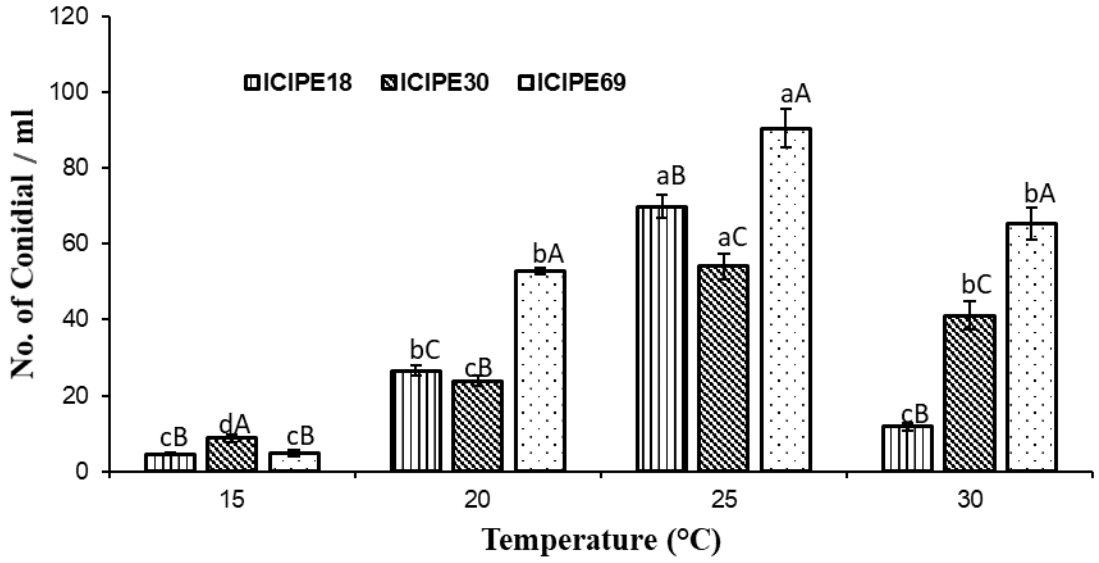


*Figure 4.1:* Effect of temperature on conidia production/sporulation of the three *Metarhizium anisopliae* isolates

## 4.6 Effect of Temperature on Virulence of *M. anisopliae* Isolates to *Z. cucurbitae*

All the three isolates were virulent against *Z cucurbitae,* with their mortality increasing from 31-100%, 16-80% and 24-96% for ICIPE 18, ICIPE 30 and ICIPE 69 respectively across the temperature regimes of 15 – 30°C (Table 4.6). The highest mortality was caused by isolate ICIPE 18 followed by ICIPE 69 across the temperature regime of 15 – 30°C. Temperature was found to have a significant ($P<0.001$) effect on the virulence of all the three isolates (Appendix 4). The highest mortality occurred at 25°C and 30°C temperature regimes, which were not significantly different from each other. The isolates also varied significantly from each other in their virulence to melon fly at different temperatures except at 15°C where all the isolates produced similar effects (Table 4.6). There was significant interaction between the temperature and the fungal isolates ($P<0.05$) in terms of mortality rates as different isolates produced different effects at different temperature regimes.

Table 4.6: Mortality of adult *Z. cucurbitae* caused by *M. anisopliae* isolates at different temperature regimes at 4 days' post-exposure

| Temperature | Fungal isolates | | |
|---|---|---|---|
| | ICIPE 18 | ICIPE 30 | ICIPE 69 |
| 15 $^{o}$C | 31.25 ± 3.15c A | 16.25± 3.15b A | 23.75± 5.15b A |
| 20 $^{o}$C | 66.25± 3.75b A | 22.5± 3.23b B | 37.5± 8.29b B |
| 25 $^{o}$C | 98.75± 1.25a A | 76.25± 3.15a B | 96.25± 1.25a A |
| 30 $^{o}$C | 100.00 ± 0.00a A | 80.00 ± 2.04a B | 96.25± 2.39a A |
| Temperature | $F_{3,36}$ =214.76 | P<0.001 | |
| isolate | $F_{2,36}$ =56.46 | P<0.001 | |
| Temperature X isolate | $F_{6,36}$ =2.63 | P<0.05 | |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

The efficacy was also measured by the lethal time to 50% and 90% mortality ($LT_{50}$ and $LT_{90}$) values. The isolate ICIPE 30 therefore recorded the same $LT_{50}$ values at 25°C and 30°C. The $LT_{90}$ values were 3.84, 4.70 and 3.92 at 25°C reducing to 3.72, 4.54 and 3.70 at 30°C for ICIPE 18, ICIPE 30 and ICIPE 69 respectively (Table 4.7). Therefore, isolates ICIPE 18 and ICIPE 69 portrayed the highest efficacy in control of *Z. cucurbitae* within the temperature range of 25°C to 30°C.

Table 4.7: Lethal time to 50 and 90 % mortality of adult *Z. cucurbitae* caused by *M. anisopliae* isolates at different temperature regimes at 95% fiducial limit

| | **ICIPE 18** | | | **ICIPE 30** | | | **ICIPE 69** | |
|---|---|---|---|---|---|---|---|---|
| **Temperature** | $LT_{50}$ (days) | $LT_{90}$ (days) | | $LT_{50}$ (days) | $LT_{90}$ (days) | | $LT_{50}$ (days) | $LT_{90}$ (days) |
| 15°C | 4.89 (4.72-5.06) | 7.41 (7.04-7.78) | | 5.25 (5.04-5.46) | 7.00 (6.61-7.40) | | 5.40 (5.19-5.64) | 8.04 (7.56-8.52) |
| 20°C | 3.36 (3.3-3.41) | 5.07 (4.95-5.19) | | 5.32 (5.10-5.54) | 7.69 (7.26-8.13) | | 4.46 (4.34-4.58) | 6.53 (6.28-6.78) |
| 25°C | 2.71 (2.68-2.75) | 3.84 (3.78-3.9) | | 2.99 (2.94-3.04) | 4.70 (4.59-4.8) | | 2.71 (2.67-2.75) | 3.92 (3.85-3.98) |
| 30°C | 2.63 (2.59-2.66) | 3.72 (3.66-3.77) | | 2.99 (2.94-3.03) | 4.54 (4.45-4.63) | | 2.61 (2.57-2.64) | 3.70 (3.65-3.76) |

### 4.6.1 Modelling of Temperature-dependent mortality rates of adult *Z. cucurbitae.*

A non-linear regression model was used to predict the efficacy of the fungi in relation to temperature. In all the isolates tested, the quadratic model indicated that mortality of *Z. cucurbitae* increased significantly as temperature increased up to an optimum temperature range of 25°C – 30°C, beyond which the mortality started reducing (Figure

4.2). The model predicted the minimum temperatures to range between 10°C and 15°C and the maximum to be between 40°C and 45°C.

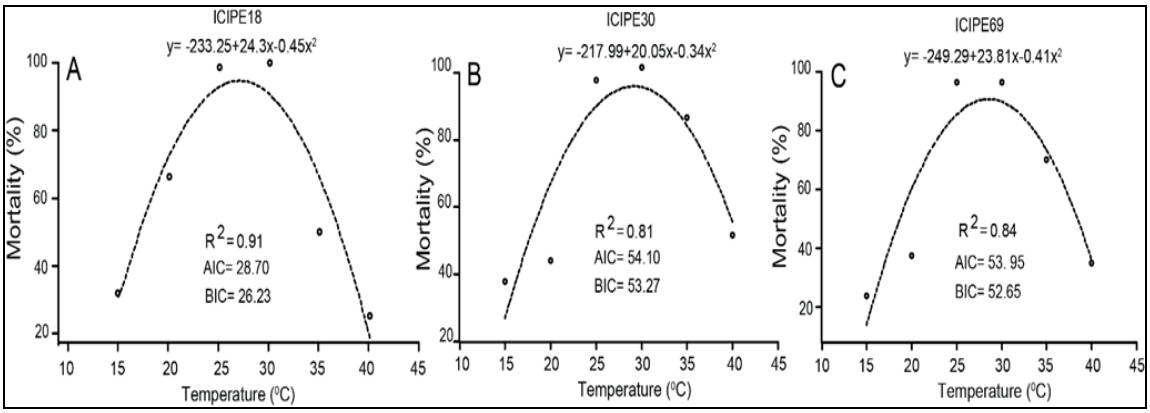


Figure 4.2: Temperature-dependent mortality rates of adult *Zeugodacus cucurbitae.* Markers are observed mean mortalities. AIC is Akaike information criterion and BIC is Bayesian information criterion.

From the three isolates, the data on percentage mortality was used for the global prediction of mortality using the geospatial temperature data layer and the best fitted quadratic model. ICIPE 18 and ICIPE 69 were potential isolates but ICIPE 69 was superior in terms of sporulation at most of the temperatures tested as compared to ICIPE 18 and was therefore selected for global prediction as a representative. The global prediction of mortality for ICIPE 69 is shown in Figure 4.3. Four colours were used to indicate the strength of the prediction. The map shows that the fungus would be most effective in the tropical climates of Africa and South America and least effective in Asia, Canada and United States of America.

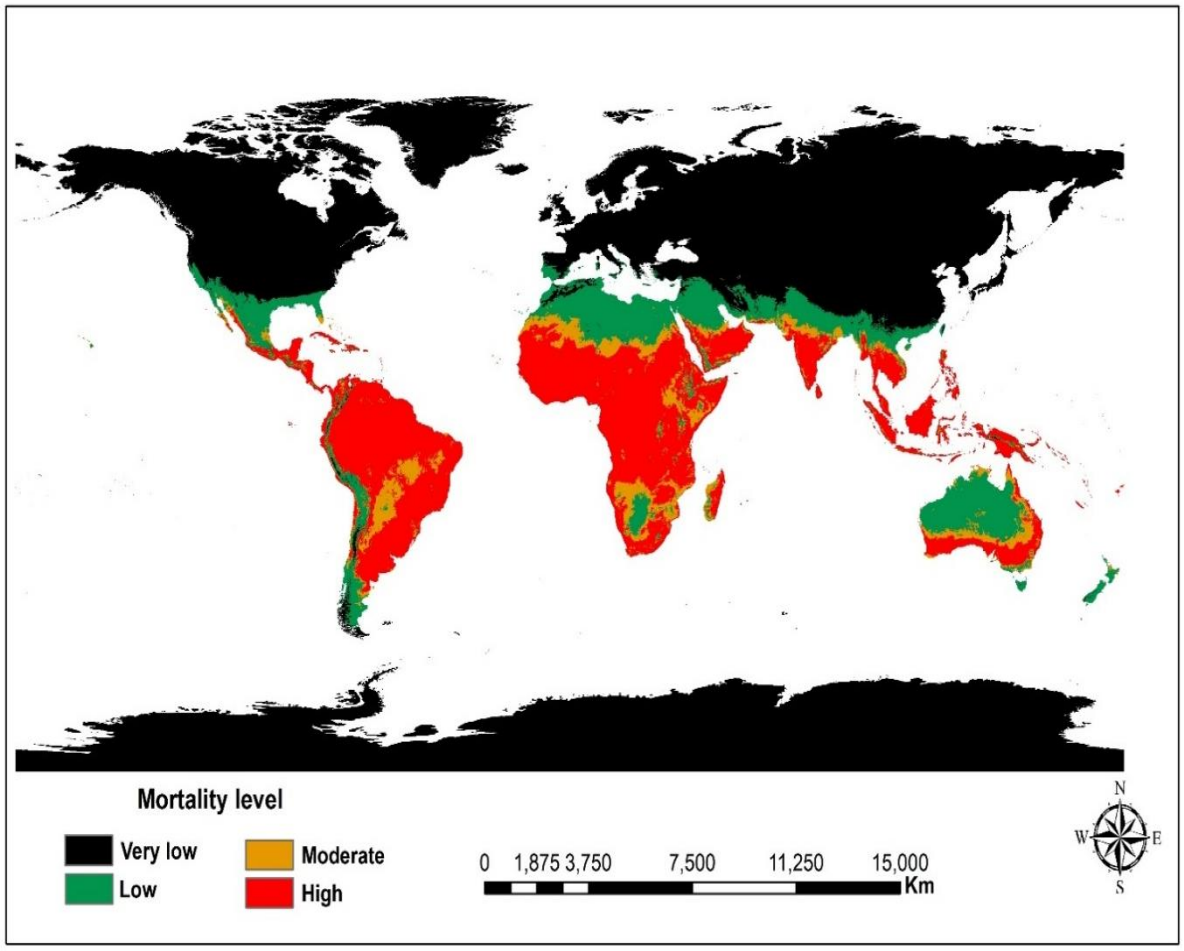


Figure 4.3: Global map predicting the efficacy of *M. anisopliae* isolate ICIPE 69 against *Z. cucurbitae*.

## 4.6 Compatibility of ICIPE 69 with Cue-lure

Conidial germination and germ tube length were used to establish the compatibility of the selected isolates with the available commercial lure.

### 4.7.1 Effect of Cue-lure on conidial germination of *M. anisopliae* ICIPE 69 at different temperatures

The germination of conidia of *M. anisopliae* was significantly affected by temperature ($P<0.001$) and period of exposure ($P<0.001$) as shown in Table 4.8. However, there was no significant interaction between exposure period and temperature ($P>0.05$) (Appendix 5). Significant reduction in conidial germination was observed in all the treatments for all the days (Table 4.8).

### 4.7.2 Effect of Cue-lure on Germ Tube Length of *M. anisopliae* ICIPE 69 at different temperatures

The germ tube length of *M. anisopliae* was also significantly affected by temperature ($P<0.001$) and period of exposure ($P<0.001$) (Table 4.9). However, there was no significant interaction between exposure period and temperature ($P>0.05$) (Appendix 5). Significant reduction of germ tube length was observed in all the treatments from day 1 to day 8 (Table 4.9).

Table 4.8: Effect of Cue-lure on *M. anisopliae* conidial germination (%) over time

| Temperature | Days after Exposure | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 6 | 8 |
| 18°C + Cue-lure | 80.60±0.79f A | 80.42±1.41f A | 82.29±1.42b A | 81.75±2.95cd A | 71.05±2.85cd B |
| 18°C control | 98.10±0.80a A | 97.90±0.37ab A | 92.11±0.62abc A | 72.27±2.95cd B | 69.69±1.49d B |
| 25°C + Cue-lure | 92.09±1.65cd A | 89.69±0.82c A | 83.44±0.66bcd B | 77.19±0.81bc C | 68.91±0.6d D |
| 25°C control | 98.46±0.25a A | 96.26±0.52ab A | 95.81±1.62a A | 93.88±0.99a AB | 88.93±2.05a B |
| 30°C + Cue-lure | 97.56±0.96ab A | 96.13±0.92ab A | 93.51±0.46ab AB | 76.90±2.88bc C | 87.84±0.38a B |
| 30°C control | 99.12±0.08a A | 98.64±0.17a A | 96.32±0.45a AB | 93.44±0.27a B | 78.19±1.51bc C |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

Table 4.9: Effect of Cue-lure on *M. anisopliae* mean conidial germ tube length (μm) over time

| Temperature | Days after Exposure | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 6 | 8 |
| 18°C + Cue-lure | 102.65±5.13d A | 89.56±7.22de A | 56.53±3.80d B | 37.92±0.9e BC | 27.15±1.59h C |
| 18°C control | 147.16±12.82a A | 119.78±6.63abc AB | 110.61±6.42ab AB | 83.05±19.01abcd B | 75.52±4.8cd B |
| 25°C + Cue-lure | 122.38±2.88abcd A | 88.62±6.05de B | 62.47±4.64d C | 56.73±1.43deC | 35.28±3.52gh D |
| 25°C control | 113.26±2.28cd A | 103.09±0.92cde AB | 96.30±3.02bc BC | 84.80±2.04abcd CD | 74.91±4.79cd D |
| 30°C + Cue-lure | 123.19±3.48abcd A | 119.59±4.62abc A | 103.13±3.97b B | 91.55±2.21abc BC | 85.59±1.38bc C |
| $30^0$C control | 145.33±5.93ab A | 140.64±0.86a A | 97.25±10.96bc B | 96.78±2.4ab B | 91.48±1.56ab B |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at $P \leq 0.05$.

### 4.8 Horizontal Transmission

#### 4.8.1 Conidia Retention

Time did not affect the retention of spores (P>0.05). However, the average number of spores picked up by a single fly immediately after exposure (0 h) was the highest. Although the number of spores reduced over time, there was no significant difference at 0 and 24 hours (Table 4.10).

Table 4.10: Number of *M. anisopliae* Conidia Retained over Time on *Z. cucurbitae* Flies

| Time after Treatment (h) | Mean number of conidia per fly (x $10^5$) |
|---|---|
| **0** | 14.4 ± 3.27 |
| **2** | 12.6 ± 1.33 |
| **4** | 12.5 ± 2.24 |
| **6** | 9.45 ± 2.11 |
| **8** | 6.55 ± 1.62 |
| **24** | 6.41 ± 0.87 |

**Key:** Means within a column followed by the same lower case letter and within a row followed by the same upper case letter are not significantly different at P ≤ 0.05.

#### 4.8.2 Horizontal transmission of ICIPE 69 between *Z. cucurbitae* adults

Both "donor" and "recipient" flies acquired and succumbed to death due to fungal infection. The "donors" had 100% mortality with a less lethal time values compared to the "recipients" who had mortalities of 97.25% ± 1.6 and 86% ± 5.85 for male and female respectively. There was however, no significant difference in mortality between the donors and recipients (P>0.05).

Table 4.11: Percentage mortality and lethal time values of *Z. cucurbitae* after exposure to *Metarhizium anisopliae*

| | % Mortality ± SE | LT 50 | LT 90 |
|---|---|---|---|
| **Male 'donor'** | 100 | 2.52 (2.48–2.55) | 3.51 (3.46–3.56) |
| **Female 'donor'** | 100 | 2.64 (2.61–2.68) | 3.61 (3.56–3.66) |
| **Male 'recipient'** | 97.25 ± 1.6 | 4.45 (4.40–4.50) | 6.83 (6.76–6.92) |
| **Female 'recipient'** | 86 ± 5.85 | 7.03 (6.95–7.11) | 11.25 (11.05–11.46) |

### 4.8.3 Effect of *M. anisopliae* on Reproduction Potential of *Z. cucurbitae*

Infection by *M. anisopliae* significantly affected egg laying of melon fly. More eggs were laid by fungus-free female flies than by fungus-treated ones ($F = 147$; $df = 1, 38$; $P < 0.001$) (Figure 4.4). However, no significant difference in the hatchability of eggs was observed between the eggs from fungus-treated flies and untreated controls. ($P > 0.05$) (Figure 4.5).

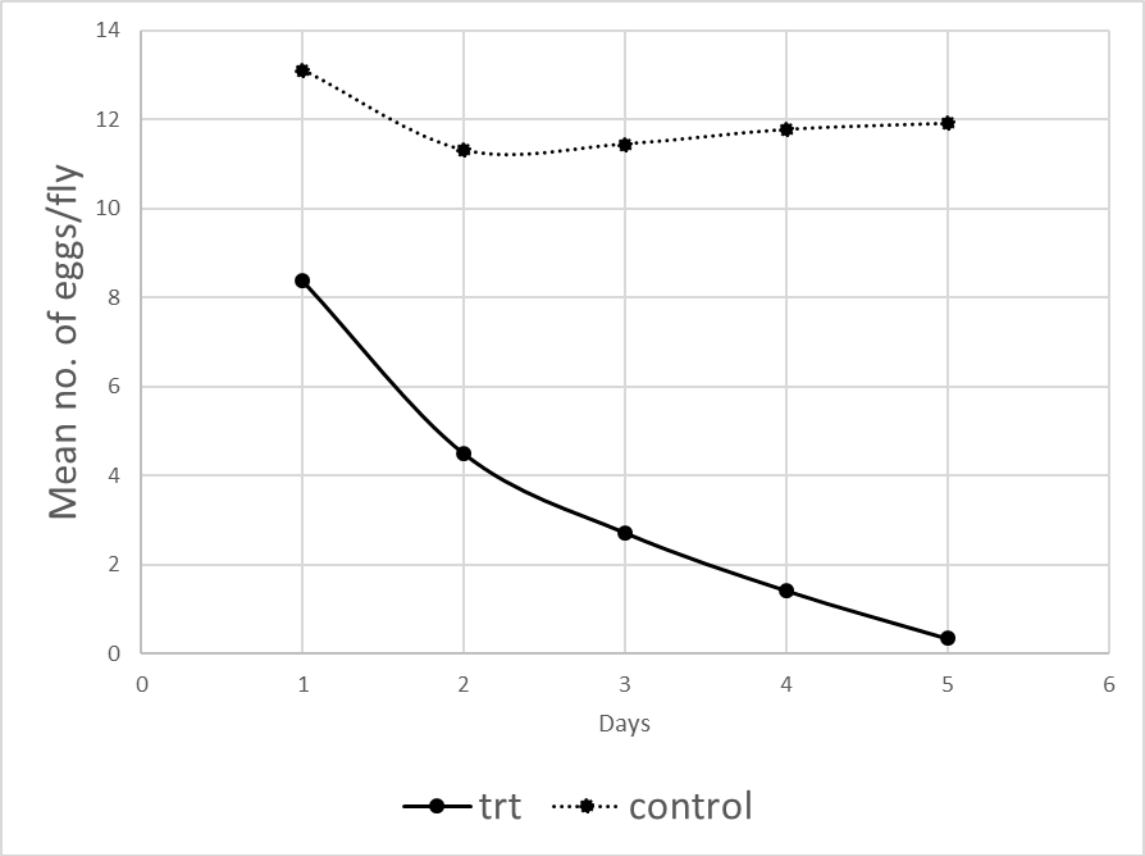


Figure 4.4: Effect of *Metarhizium anisopliae* ICIPE 69 on reproduction potential of *Zeugodacus cucurbitae*

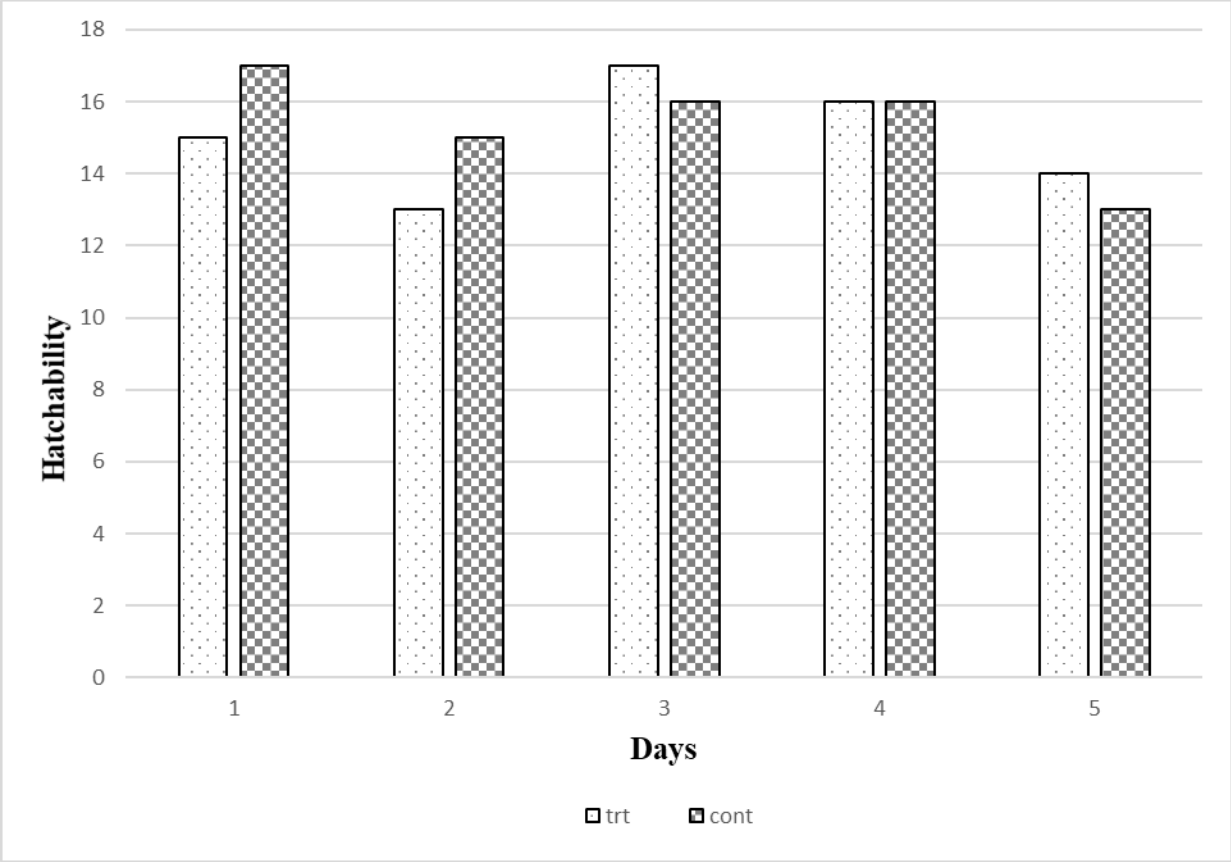


Figure 4.5: Effect of *Metarhizium anisopliae* ICIPE 69 on hatchability of *Zeugodacus cucurbitae*

# CHAPTER FIVE

# DISCUSSION

## 5.1 Pathogenicity of EPF isolates against *Zeugodacus cucurbitae* adults

This study mainly focused on identifying the most efficient entomopathogenic fungal isolate for the management of *Z cucurbitae* which is a serious pest of cucurbits. The bioassay results showed that all the 15 isolates tested were pathogenic to *Zeugodacus cucurbitae*. Other previous studies have also reported susceptibility of fruit flies to entomopathogenic fungi (Rtu *et al*.,2009; Oreste *et al*., 2015; Gul *et al*., 2015; Qazzaz *et al*., 2015). The virulence of different fungal isolates has also been reported on other pests such as *Aphis craccivora* (Mweke *et al*., 2018), *Spoladea recurvalis* moths (Opisa *et al*., 2018), Pea Leafminer (Migiro *et al*., 2010) and Leaf Beetle *Agelastica alni* (L.) (Sonmez *et al*., 2017). The results of this study confirm the pathogenicity of *B. bassiana* and *M. anisopliae* towards adult fruit flies. Isolates of *B. bassiana* were the least virulent against the pest compared *to M anisopliae* isolates. The pathogenicity of *M. anisopliae* has been previously reported against other fruit fly species like *C. rosa* var. *fasciventris*, *C. capitata* (Beris and Papachristos, 2013) and *C. cosyra (Dimbi et al., 2003; Sookar et al*.,2008). *M. anisopliae* isolates ICIPE 69 and ICIPE 18, which were found to be the most virulent against both the 3$^{rd}$ instar larvae and adults of *Z. cucurbitae* in this study, have also been reported to be effective against aphid pest species of crucifers and okra (Bayissa *et al*., 2017), western flower thrips, *Frankliniella occidentalis* (Niassy *et al*., 2012) and *Aphis craccivora* (Mweke *et al*., 2018).

The present study employed the use of velvet material contaminated with dry conidia to inoculate the insects as previously used by Migiro *et al*. (2010) and Dimbi *et al*. (2013). Due to this reason, it was therefore not possible to determine the Lethal Concentration LC values for the most virulent isolates (Bayissa *et al*., 2017). The difference in virulence among the isolates of *M. anisopliae* was confirmed by the LT90 values recorded. The LT90s ranged from 3.79 days for ICIPE 69 to 6.65 days for ICIPE 279. The LT90 values for the most virulent isolates of *M. anisopliae* tested in this study ranged from 4.8 to 5.0

days. This was slightly higher than LT90 values obtained by Dimbi *et al*. (2003), which ranged from 3.3 to 4.5 days. However, it was lower than the LT50 values reported by Castillo *et al*. (2000) which ranged from 5.6 to 5.9 days, within a similar range of concentration of conidia pick. These differences were attributed to differences in the virulence of the fungus used in the different studies or the action of fungus on different species of pests.

**5.2 Exposure of 3rd instar larvae to conidial suspension**

*M. anisopliae* has been found to effectively suppress the emergence of the fruit flies when the fungus is applied to the soil and then the insects are introduced (Gul *et al*., 2015). Applications of entomopathogenic fungi prior to the introduction of pupae into the soil have been recommended (Ekesi *et al*., 2002). This is mainly because it may enhance the contact effects of the fungus (in time and with regard to coverage) as the last larval stage of *Z. cucurbitae* drop on and pupate beneath the soil surface, therefore narrowing the 'window' for infection. For soil applications of entomopathogenic fungi to be successful, factors such as pathogen virulence, soil type, application method, environmental conditions and the presence or absence of a susceptible host have to be considered (Inglis et al. 2001). In most studies, depending on the insect, it has proven difficult to have pupae as the primary target. This is because the thick and sclerotised pupal cuticle provides a great barrier to fungal infection and the inactive nature of pupae (De La Rosa *et al*. 2002). Therefore, targeting the last-instar for some pests has been found to be a good control because it results to high levels of pupal mortality. In this study, the 3$^{rd}$ instar lavae were targeted because of the life cycle of melon fly, where the other instars occur inside the fruit host and will pop out to pupate at this instar. Only a few adult melon fly emerged from the various fungal treatments, and half of these ultimately developed mycoses.

In the present study, the emergence of the flies in the control treatment was higher than in all the inoculated treatments. Similar findings were reported by Inglis *et al*. (2001). However, different inoculation concentrations did not differ significantly from each other but they were all different from the control with regard to their effect on pupa emergence.

It was therefore evident that soil inoculation had a significant effect on the adult emergence. The percentage emergence from the inoculated treatments ranged between 3% to 52% depending on the isolate and the conidial concentration. Pupae emergence values obtained in a study by Beris and Papachristos (2013), were within the range of this study. The results from this study therefore confirmed the ability of EPFs to suppress pupa emergence as reported in several previous works e.g., (Ekesi, 2002; Ekesi *et al*., 2005; Khlaywi *et al*., 2014).

There was also significantly higher mortality of insects that emerged from inoculated treatments than in the control. The highest emerged insects mortality in the three isolates was obtained at the highest concentration of $1.0 \times 10^8$. This concentration was also reported as the most effective on other bactrocera species (Mar and Lumyong, 2012). In this study, it is possible that the emerging adults acquired the inoculum after emergence as they moved through the soil. However, the time interval from emergence to death was relatively short, which could suggest that few infective propagules may have been acquired before pupation. Since mortality is dose related (level and rate), an infection took longer to develop, manifesting only in the emerged adult stage. It is possible that these individuals survived through to adulthood, but succumbed shortly thereafter due to colonizing effect of the fungi.

Since all the tested concentrations were able to reduce pupa emergence and longevity in this study, the results suggest that *M. anisopliae* fungal isolates are effective against 3rd instar stages of *Z. cucurbitae*. *Metarhizium anisopliae* has also been reported to cause 41 and 46% pupae mortality of western flower thrips and legume flower thrips, *Megalurothrips sjostedti* Trybom (Thysanoptera: Thripidae), respectively (Ekesi and Maniania, 2000). Wraight and Ramos (2004) also found that by spraying last-instar of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) with *B. Bassiana*, pupal mortality was achieved. In the present study, only insects that showed mycosis were analyzed. In all treatments, the time taken for mycosis to be seen and the mycelium density were different. This had also been observed in a study carried out by Mar and Lumyong (2012). These data demonstrate that the strains of *M. anisopliae* are a

robust and effective control agent for melon fly pupae and that conidial concentration affect the emergence and mortality which concurs with other studies in that emergence increases with decrease in concentration (Beris and Papachristos, 2013).

### 5.3 Effect of Temperature on Conidial Germination

The temperature was observed to have a significant effect on spore germination of the three selected isolates of *M. anisopliae* with the optimum temperature being 25 and 30°C. Similar results were observed by Gougouli and Koutsoumanis (2012) on *Penicilium. expansum*, and Pardo *et al*. (2005) on *Aspergillus ochraceus*. There was no significant difference among isolates with regard to spore germination except for ICIPE 69 at 15°C. This was attributed to the fact that the three isolates originated from similar environmental conditions although in different countries.

### 5.4 Effect of Temperature on Fungal Growth

Fungal growth was found to occur at all temperatures but there was minimal growth at 15°C while 30°C recorded the highest growth rate. Similar findings were reported by Tumuhaise *et al.* (2018) on ICIPE 18 and 69 isolates and Bayissa *et al*. (2017) on ICIPE 30 isolate in their thermo-tolerance studies involving *Maruca vitrata* Fabricius (Lepidoptera: Crambidae). The highest growth rate for all the three isolates was observed at 30°C but there was no significant difference between isolates in growth rates recorded at the temperature of 25°C and 30°C. Therefore, the optimal temperature for radial growth of these isolates was considered to be 25°C and 30°C. This was within the optimum range reported in other entomo-pathogenic studies (Ouedraogo *et al*., 1997). The highest growth rate observed among the isolates ranged from 3.79 to 4.08 which was within the range observed by Cabanillas and Jones (2009) on most *Isaria* isolates. The similar behavior of the isolates observed within the temperature range of 25°C and 30°C was attributed to the fact that they were all isolated from the soil or from a soil organism under tropical conditions.

### 5.5 Effect of Temperature on Fungal Sporulation

There was a significant effect of temperature on sporulation of all the isolates. The optimum sporulation temperature was found to be 25°C with a significant reduction of

conidia at 30°C. A study by Chauvet and Suberkropp (1998) on aquatic Hyphomycetes showed that *L. curvula* and *T. marchalianum* sporulated at an optimum temperature of 25°C, which is in agreement with this study. These findings also corroborate the findings of a similar study by King *et al*., (2007) on *Colletotrichum Spp*. In this study, the optimum temperature for sporulation was found to be lower than that of radial growth. This could mean that the sporulation of the selected isolates is heat sensitive and hence the sporulation started to decrease with increase of temperature after 25°C.

### 5.6 Effect of Temperature on Virulence of *M. anisopliae* Isolates to *Z. cucurbitae*

The virulence bioassay showed that mortality of *Z. cucurbitae* adults caused by *M. anisopliae* isolates was significantly affected by the temperature. Other studies that reported similar results with *M. anisopliae* isolates include Yeo *et al*. (2003) and Bayissa *et al*. (2017) on aphid species, Ugine (2011) on *Lygus lineolaris* and Mishra *et al*. (2015) on *Musca domestica* L. They all reported that virulence of *M. anisopliae* isolates increased with increase in temperature. The isolates ICIPE 18 and 69 caused significantly higher mortality than ICIPE 30 across the temperature range of 20°C and 30°C. This could be attributed to the fact that they were both soil isolates and were both isolated from tropical regions though in different countries. Similar results with the same isolates were reported by Bayissa *et al*. (2017) on aphid species. The optimum temperature at which the highest mortality occurred for all the isolates ranged from 25°C and 30°C. This finding was in agreement with that of Bayissa *et al*. (2017) who observed that *M. anisopliae* isolates were more virulent to aphid species at 25°C and 30°C than at 15°C and 20°C. The isolates ICIPE 18 and 69 also recorded the shortest lethal time within the optimal virulence temperature of 25°C and 30°C. This therefore showed that the two isolates would have the highest efficacy in control of *Z. cucurbitae* within its optimum development temperature range of 25°C to 32°C (Maynard, 2007).

#### 5.6.1 Modelling of Temperature-dependent Mortality Rates of Adult *Z. cucurbitae*

According to Klass et al. (2007), modelling is important as it provides a useful tool to assist in interpreting effectiveness of control operations which leads to development of improved application strategies to optimize the performance of the bio-pesticide. In this

study, the non-linear regression model was used to predict the efficacy of the fungi in relation to temperature. The minimum threshold temperatures for fungal isolates efficacy were estimated by the quadratic equation to be between 10-15°C, the optimum to be between 25°C to 30°C and the maximum to be between 40-44$^0$C. These estimates were within the range of values obtained by Rangel *et al*. (2010). At optimum temperatures of 25°C to 30°C, ICIPE 18 and 69 consistently produced similar results in germination, radial growth and percentage mortality. ICIPE 18 showed tolerance over a broad range of temperatures including the lower temperatures of 15°C to 20°C. However, ICIPE 69 outperformed other isolates in suppression of pupa emergence and mortality, sporulation and lower LT values. This isolate was therefore selected for global mapping to predict its efficacy against *Z. cucurbitae* using the geospatial temperature data layer and the best fitted quadratic model. The global map showed that ICIPE 69 would be more effective in the tropics than the temperate regions, which is in agreement with Tumuhaise *et al*. (2018). This isolate could therefore be integrated with other control agents such as use of Cue-lure pheromone food bait (Street *et al.,* 2016) and other cultural practices (Dhillon *et al*., 2005; Ryckewaert *et al*., 2010).

### 5.7 Compatibility of ICIPE 69 with Cue-lure

The Cue-lure evaluated in this study was compatible with the isolate ICIPE 69. Cue-lure can therefore be synergistically used with the fungal isolates for effective control of *Z. cucurbitae* where the lure would attract the insect while the fungal isolates would be the bait to kill the insect. However, the influence of temperature on compatibility was significant. In addition, the period after exposure was also of significant influence and conidial germination and germ tube length reduced over time (David *et al*., 2016). Cue-lure may possibly have some antagonistic effects to the fungus. Antifungal effects have been reported in other attractants (Halim *et al.,* 2006; Erdemgil *et al*., 2007; Chambers *et al*., 2013). Higher conidial germination and longer germ tube length has been found to directly influence fungal pathogenesis on insects. Fargues *et al*. (1994) compared four different growth stages of *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) (Eurotiales: Trichocomaceae) for their infection potential to the first-instar larvae of *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae) and found germinated conidia and hyphal

bodies to be more aggressive than ungerminated conidia. Similar findings were also reported by Faria *et al*. (2015).

### 5.8 Horizontal Transmission

For a bio-control agent to be effective, the dispersal should be efficient and still cause acute mortality. Horizontal transmission can be achieved through avenues like mating or physical contact. Flies generally do a lot of grooming after they have been exposed to fungus but the fact that flies were able to retain substantial amount of conidia after grooming has important implications on the horizontal transmission. In this study, when fungus-treated fruit flies (donors) were mixed with healthy flies (recipients), they were able to transmit fungal conidia to the healthy ones, thus, causing mortality of up to 97% in 10 days after inoculation. This study therefore demonstrated that horizontal transmission of fungal infection does occur during mating and physical contacts and that *M. anisopliae* may have potential for the integrated control programs of *Z. Cucurbitae.* A study by Aanen and Nobre (2010) identified horizontal transmission as key to the colonization of Madagascar by fungus-growing termites. Several studies have also shown effectiveness of horizontal transmission of the entomopathogenic fungus e.g. *Beauveria bassiana* against *Triatoma infestans* (Forlani and Pedrini, 2011), *totivirus* in violet root rot fungus *Helicobasidium mompa* (Suzaki and Atsuko, 2005), hypoviruses between vegetative compatibility types of *Cryphonectria parasitica* in Macedonia (Papazova *et al*, 2008), *Isaria fumosorosea* against whitefly (Avery *et al*., 2010) and selected Brazilian strains of *Beauveria bassiana* against *Cosmopolites sordidus*.

In tephritid fruit flies, effective horizontal transmission has also been reported in several studies. For example, Toledo *et al.* (2012) reported effective horizontal transmission of *Beauveria bassiana* in *Anastrepha ludens* (Diptera: Tephritidae) under laboratory and field cage conditions and Quesada-Moraga *et al*. (2008) on *C. capitata* (Wiedemann) (Diptera: Tephritidae). Effect of *M. anisopliae* inoculation on the mating behavior of three species of African Tephritid fruit flies, *C. capitata*, *C. cosyra* and *C. fasciventris* was reported by Dimbi *et al*. (2009 and 2013) where the fungus caused significant

reduction in the number of eggs oviposited and fecundity was reduced to as low as 37% in *C. cosyra*.

However, no significant difference on the hatchability of eggs was found between fungus treated flies and untreated controls. This could mean that the viability of the egg that is oviposited by an infected fly is not affected. This findings from this study corresponds with those of Dimbi *et al*. (2013) on the three species of fruit flies namely *C. capitata, C. cosyra* and *C. fasciventris*.

# CHAPTER SIX

# SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

## 6.1 Summary of findings

The key findings of this study can be summarized as follows:

1. *Metarhizium anisopliae* isolates showed potential in the management of *Z. cucurbitae*, as compared to *B. bassiana* isolates. *Metarhizium anisopliae* isolate ICIPE 69 and ICIPE 18 were the most promising candidates for the control of *Z. cucurbitae* on cucurbits as determined by their relatively higher ability to suppress pupae emergence and subsequent mortality of the emerged pupae from contaminated soil.
2. Temperature was found to have a significant effect on conidia germination, radial growth, sporulation and pathogenicity of the three tested isolates of *M. anisopliae*. This indicated that pathogenicity of the isolates was temperature dependent. The optimum temperature for conidia germination, radial growth, sporulation and virulence was 25 and 30°C. *Metarhizium anisopliae* isolates ICIPE 69 and ICIPE 18 again recorded the highest conidia germination, radial growth, sporulation and pathogenicity against *Z. cucurbitae* among different temperature regimes.
3. *Metarhizium anisopliae* isolate ICIPE 69 was found to be compatible with a commercially available Cue-lure. However, the influence of temperature on compatibility was significant. In addition, the period after exposure was also of significant influence and conidial germination and germ tube length reduced over time. The study showed that there was an effect of Cue-lure on fungus
4. Although the flies that picked conidia from the contamination devices were likely to lose some of it during flight, infected flies were able to infect healthy flies causing mortality. This study therefore demonstrated that horizontal transmission of fungal infection from infected to heathy flies occurred and was effective in reducing the population of the pest.

## 6.2 Conclusions

Based on the study objectives, the following conclusions were drawn from the study findings:

1. *Metarhizium anisopliae* isolates ICIPE 18, ICIPE 30 and ICIPE 69 are highly pathogenic against the adults of *Z.cucurbitae*. The isolates can also reduce the emergence of pupae and reduce the longevity of emerged flies. However, for optimum results, the right concentration should be used. A concentration of 1 x $10^8$ spores/ml was found to be very effective.
2. Temperature has a profound influence on the performance of the selected isolates on conidial germination, sporulation, radial growth and virulence. The optimal temperature for the best performance was 25 and 30°C. However, *M. anisopliae* isolate ICIPE 69 and ICIPE 18 can be effective used as entomopathogens against the melon fruit fly (*Z. cucurbitae*). The isolates can thus be further developed and used within the framework of IPM programs in the field. The global mapping to predict the efficacy against *Z. cucurbitae* using the geospatial temperature data layer and the best fitted quadratic model showed that ICIPE 69 would be more effective in the tropics than the temperate regions. This is deduced to be the same case with isolate ICIPE 18. This study therefore demonstrates the potential of the two *M. anisopliae* isolates as candidates of biological control agents for *Z. cucurbitae* in the tropics.
3. This study showed that Cue-lure is compatible with conidia of *M. anisopliae* ICIPE 69. The fungus and lure can therefore be used together in the auto dissemination device for “lure and kill” management strategy.
4. This study demonstrated that horizontal transmission of fungal infection does occur during mating and physical contacts and is effective in reducing the population of the pest. The effectiveness of the horizontal transmission in reducing the pest population is further supported by the fact that fungus-infected female flies laid fewer eggs than healthy flies although the infection did not have any effect on the fertility of the eggs. Therefore, *M. anisopliae* may have some considerable potential in the integrated control programs of *Z. cucurbitae*.

## 6.3 Recommendations

The following recommendations were made from the study:

1. Since ICIPE 69 is already commercialized, *Z. cucurbitae* can be included in the list of the pests controlled by this isolate.
2. Since the virulence of the selected *M. anisopliae* isolates was found to be temperature dependent, the optimum temperature of 25 and 30°C is recommended for application of these isolates in melon fly management in order to obtain the best results.
3. Based on the laboratory observations, the *M. anisopliae* isolate, ICIPE 69 was found to be compatible with commercially available pheromone lure "Cue-lure" for the management of *Z. cucurbitae.* The isolate is therefore recommended to the manufacturers of pheromone lures for incorporation as a bait.
4. Since the infected flies were found to effectively infect healthy flies and subsequently cause high fly mortality, entomopathogenic use of the two selected *M. anisopliae* isolates (ICIPE 69 and 18) is highly recommended in the IPM package for control of *Z. cucurbitae*.

## 6.4 Areas of Further Research

Based on the study findings, the following areas of further research are suggested:

1. Since different agrochemicals are used for the management of melon fly and other pests of cucurbits, effects of these chemical insecticides to *M. anisopliae* isolates, particularly ICIPE 69 and 18, should be undertaken.
2. To ascertain the compatibility of the fungal isolates and commercially available Cue-lure, the laboratory findings should be confirmed through a field based experiment.
3. Although the effectiveness of ICIPE 69 fungal isolate was tested at different temperatures and found to be most effective in the temperature range conducive for melon production, the isolate should also be evaluated under field conditions. This is important because there are many other factors that may come into play under field conditions including humidity and fluctuations of day and night temperatures.

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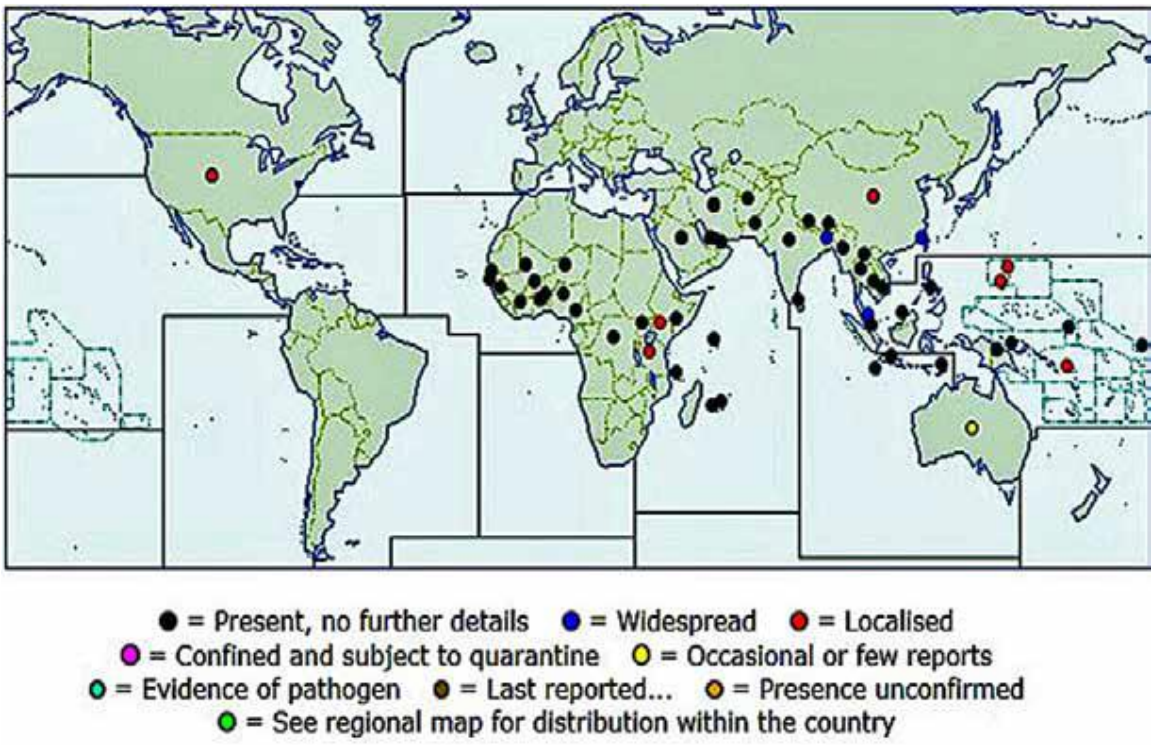
**APPENDICES**

Appendix 1: Geographical Distribution of Melon Fly, *Z. cucurbitae*



Appendix 2: Summary of the main reports of insecticide resistance mechanisms in Tephritidae

| **Species** | **Strain Origin** | **Insecticide (active ingredient)** | **RR[c]** |
|---|---|---|---|
| *B. oleae* | L. Greece | OP, dimethoate | 10 |
| | F. Greece | OP, dimethoate | 64 |
| | F. Greece | PYR, alpha-cypermethrin | 64 |
| | F. California | SPI, spinosad | 9-13 |
| | F. Greece | SPI, spinosad | 2-4 |
| *B. dorsalis* | L, Taiwan | OP, fenitrothion | 406 |
| | L, Taiwan | OP, fenthion | 37 |
| | L, Taiwan | OP, naled | 5 |
| | L, Taiwan | PYR, fenvalerate | 131 |
| | L, Taiwan | SPI, spinaoid | 408 |
| | L, Taiwan | OP, fenitrothion | 53 |
| | F, Taiwan | OP, fenthion | 8.2 |
| | F, Taiwan | OP, naled | 6 |
| | F, Taiwan | CARB, methomyl | 33 |
| | F, Taiwan | PYR, cypermethrin | 11 |
| | F, Taiwan | PYR, cyfluthrin | 14 |
| | F, Taiwan | PYR, fenvalerate | 5 |
| | F, Taiwan | SPI, spinosad | 1-5 |
| | F, Hawaii | SPI, spinosad | 1 |
| *B. cucurbitae* | F, Taiwan | OP, dichlorvos | 1 |
| | F, Taiwan | OP, dichlorvos | 4 |
| | F, Taiwan | OP, malathion | 23 |
| | F, Taiwan | CARB, methomyl | 8 |
| | F, Taiwan | PYR, cyfluthrin | 29 |
| | L, Taiwan | OCL, lindane | 16 |
| *C. capitata* | L, Spain | OC, DDT | 15 |
| | L, Spain | OC, dieldrin | 24-67 |
| | L, Spain | OP, malathion | 3 |
| | L, Spain | OP, dimethoate | 2 |
| | L, Spain | PYR, Lambda-cyhalothrin | 42 |
| | F, Spain | OP, malathion | 6-201 |

[a]Strain Origin: F - Strain obtained from field population; L - Strain obtained after laboratory selection. [b]Insecticide classes: OCL - organochlorines; OP - organophosphates; CARB - carbamates; PYR - pyrethroids; SPI - spinosad. [c]RR (Resistance Ratio) - ED50 of sample tested/ED50 of the laboratory susceptible strain.
Source: Vontas *et al*., 2011.

Appendix 3: ANOVA for Objective 1

| Trait | Source | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| Spore germination | Trt | 14 | 424.0 | 30.29 | 2.738 | 0.0101 * |
| Mortality | Trt | 14 | 35595 | 2542.5 | 25.31 | <2e-16 *** |
| Adult Emergence | Conc | 3 | 10097 | 3366 | 18.873 | 4.56e-07 *** |
| | Isolate | 2 | 653 | 326 | 1.831 | 0.178 |
| | Conc: Isolate | 4 | 812 | 203 | 1.139 | 0.357 |
| Emerged adults mortality | Conc | 3 | 10261 | 3420 | | 3.33e-05 *** |
| | Isolate | 2 | 858 | 429 | | 0.2506 |
| | Conc: Isolate | 4 | 3563 | 891 | | 0.0336 * |
| | | | | | | |

Appendix 3: ANOVA for Objective 2

| Trait | Source | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| Sporulation | Temperature | 3 | 26602 | 8867 | 362.22 | < 2e-16 *** |
| | Isolate | 2 | 2725 | 1362 | 55.65 | 2.68e-11 *** |
| | Temp: Isolate | 5 | 3757 | 751 | 30.69 | 1.67e-11 *** |
| Germination | Temperature | 3 | 41473 | 13824 | 3082.664 | <2e-16 *** |
| | Isolate | 2 | 11 | 6 | 1.227 | 0.305 |
| | Temp: Isolate | 6 | 42 | 7 | 1.570 | 0.184 |
| Radial Growth | Temperature | 3 | 54.96 | 18.319 | 241.712 | < 2e-16 *** |
| | Isolate | 2 | 2.01 | 1.005 | 13.267 | 4.82e-05 *** |
| | Temp: Isolate | 6 | 0.91 | 0.152 | 2.006 | 0.0904 |
| Mortality | Temperature | 3 | 22893 | 7631 | 214.761 | < 2e-16 *** |
| | Isolate | 2 | 4012 | 2006 | 56.455 | 7.96e-12 *** |
| | Temp: Isolate | 6 | 562 | 94 | 2.634 | 0.032 * |

Appendix 3: ANOVA for Objective 3

| Trait | Source | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|---|
| Germination | Day | 4 | 4898 | 1224.6 | 27.81 | 9.43e-16 *** |
| | Temp | 2 | 1678 | 838.9 | 19.05 | 8.72e-08 *** |
| | Day: Temp | 8 | 458 | 57.2 | 1.30 | 0.252 |
| Germ tube length | Day | 4 | 54133 | 13533 | 23.604 | 6.13e-14 *** |
| | Temp | 2 | 9567 | 4784 | 8.343 | 0.000434 *** |
| | Day: Temp | 8 | 3621 | 453 | 0.790 | 0.612956 |