IMPACT OF *Metarhizium anisopliae* AND *Beauveria bassiana* ON BEE POLLINATORS (HYMENOPTERA: APIDAE), AND MODELLING THEIR PERFORMANCE IN BEE COLONIES

Evanson Rigan Omuse

(BSc. Microbiology & Biotechnology)

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY

DEPARTMENT OF BIOLOGY

COLLEGE OF BIOLOGICAL AND PHYSICAL SCIENCES

UNIVERSITY OF NAIROBI

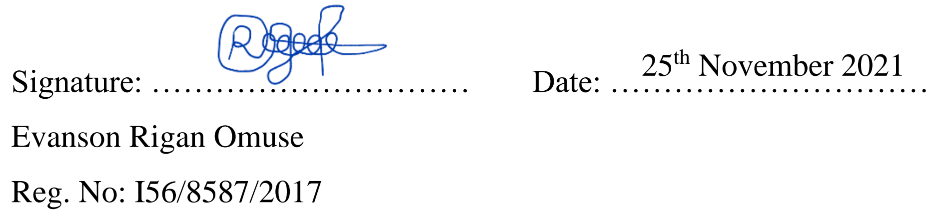
NAIROBI, KENYA

November 2021

DECLARATION

Candidate

This is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has properly been acknowledged and referenced as per the University of Nairobi's requirements.



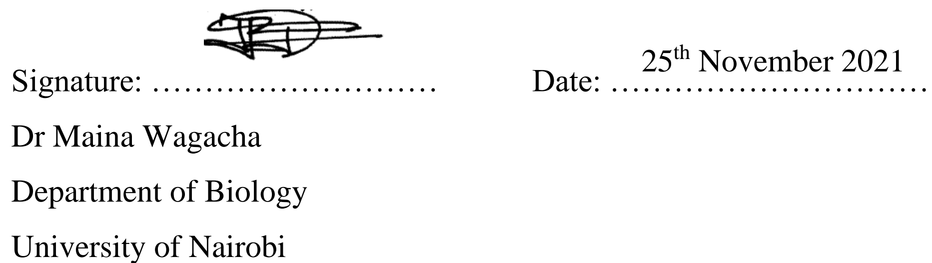
Signature: ………………………… Date: 25th November 2021

Evanson Rigan Omuse

Reg. No: I56/8587/2017

Supervisors

We confirm that the candidate undertook this research study under our supervision:

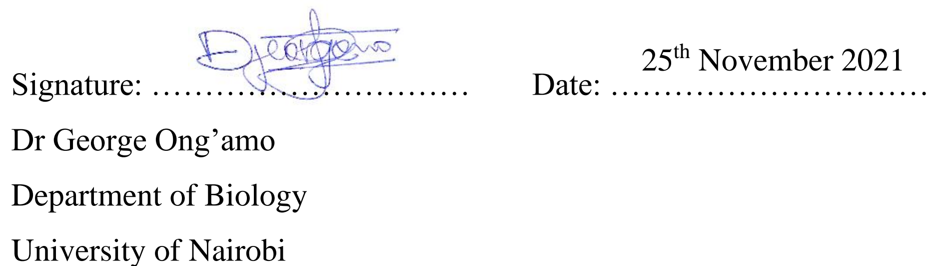


Signature: ……………………… Date: 25th November 2021

Dr Maina Wagacha

Department of Biology

University of Nairobi

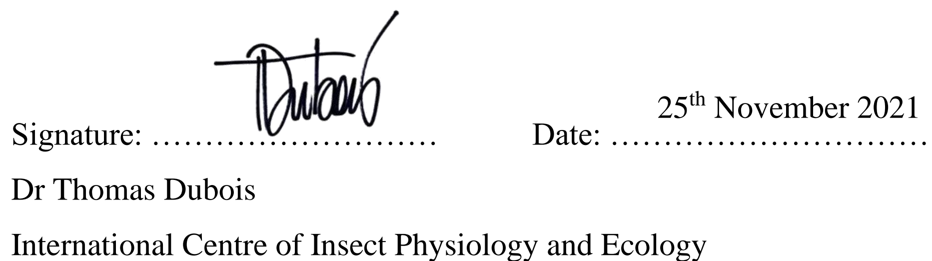


Signature: ………………………… Date: 25th November 2021

Dr George Ong'amo

Department of Biology

University of Nairobi



Signature: ……………………… Date: 25th November 2021

Dr Thomas Dubois

International Centre of Insect Physiology and Ecology

DEDICATION

This thesis is dedicated to my mother; Mrs Beatrice Kanda, brothers; Mr Patrick and George, sisters; Mrs Irine, Elizabeth and Cynthia and friends who have exhibited incredible support throughout my studies.

ACKNOWLEDGEMENT

I extend my sincere gratitude and appreciation to my supervisors at the University of Nairobi, Dr Maina Wagacha and Dr George Ong'amo for consistent guidance, support and patience during the course of this study. Equally, I appreciate my supervisors at the International Centre of Insect Physiology and Ecology (*icipe*), Dr Thomas Dubois and Dr Saliou Niassy for technical and resourceful guidance throughout the study period. I am grateful to Dr Brian Isabirye, Dr Sevgan Subramanian and Dr Samira Mohamed at *icipe* for their intellectual advice and recommendations at the beginning of this study. I acknowledge the assistance of Dr Michael Lattorff and Dr Nkoba Kiatoko for the guidance and provision of bee colonies used in this study.

Special thanks go to Mr Levi Ombura at *icipe* Arthropod Pathology Unit (APU) for his support and assistance in logistics for some of the research materials during my entire research period at *icipe*. I appreciate Mrs Gilvian Onsomu, Mr Kilonzo Wambua and Mr James Ng'ang'a for taking care of bee colonies used in this study. I appreciate Mr Joseph Ambajo, Mr Francis Odari, Mr Kevin Kiprotich, Mr Maurice Musonye and Mr Gasper Kirui for assistance in data collection during greenhouse studies. Not forgetting the *icipe* management and all the personnel of the *icipe* APU, particularly the technicians; Mrs Jane Kimemia, and Mr Osospeter Wafula, thank you. I would like to acknowledge the Federal Ministry for Economic Corporation and Development (BMZ) through the "Integrated Pest and Pollinator Management (IPPM) in avocado–cucurbits production systems in Kenya and Tanzania" project for supporting this research. I acknowledge the U.K.'s Department for International Development (DFID), Swedish International Development Cooperation Agency (Sida) and the Swiss Agency for Development and Cooperation (SDC) through *icipe* Capacity Building for providing me with an opportunity to conduct this study under the support of the Dissertation Research Internship Programme (DRIP). I acknowledge the Department of Biology for providing me with an opportunity to pursue my Master of Science study under the support of the University of Nairobi scholarship.

TABLE OF CONTENTS

Declaration ii
Dedication iii
Acknowledgement iv
List of figures viii
List of tables ix
Abbreviations and acronyms x
Abstract xii
Chapter One 14
1.0. General introduction 14
1.1. Background to the study 14
1.2. Statement of the problem and justification of the study 16
1.3. Objectives 18
1.3.1. Broad objective 18
1.3.2. Specific objectives 18
1.4. Research hypothesis 19
Chapter Two 20
2.0. Literature review 20
2.1. Pollinators and their ecosystem services 20
2.2. Pollination crisis and drivers to the decline of pollinators 21
2.3. Effect of chemical pesticides on pollinators 22
2.4. Entomopathogenic fungi 23
2.4.1. Overview of entomopathogenic fungi 23
2.4.2. Host selection and mode of action of entomopathogenic fungi 24
2.4.3. Entomopathogenic fungi as biological control agents of pests 26
2.5. Application, interaction, and safety of entomopathogenic fungi on bees 27
2.5.1. Management of crop pests and sensitivity of bees as pollinators 27
2.5.2. Management of bees' pathogens and safety to bees 28
2.5.3. Entomovectoring technology and safety of bees as entomovectors.. 30
2.6. Response of bees to entomopathogenic fungi 32
2.6.1. Immune response against mycotic invasion 32
2.6.2. Behavioural responses and protection against fungal invasion 32
2.6.3. Role of bee thermoregulation on mycotic invasion 33

Chapter Three 35

3.0. Safety of *Metarhizium anisopliae* Metsch. and *Beauveria bassiana* Bals. To the honey bee (*Apis mellifera* L.) and the stingless bee (*Meliponula ferruginea* Cockrell) (Hymenoptera: Apidae) in laboratory conditions 35

Abstract 35

3.1. Introduction 36

3.2. Materials and methods 38

3.2.1. Source and culturing of fungal isolates 38

3.2.2. Assessment of conidial germination 40

3.2.3. Preparation of fungal suspensions 41

3.2.4. Source and *in vitro* maintenance of *Apis mellifera* 41

3.2.5. Source and *in vitro* maintenance of *Meliponula ferruginea* 43

3.2.6. Exposure of caged bees to entomopathogenic fungal isolates 44

3.2.7. Assessment of conidial acquisition by the bees 44

3.2.8. Assessment of the effect of entomopathogenic fungi on bees 45

3.2.9. Data analysis 46

3.3. Results 48

3.3.1. Conidial acquisition by bees 48

3.3.2. Post-exposure survival of bees 48

3.3.3. Time-response mortality of fungus-exposed bees 52

3.3.4. Mycosis of fungus-exposed bees 53

3.3.5. Correlation of conidial acquisition with pathogenicity of fungi 54

3.4. Discussion 56

Chapter Four 60

4.0. Modelling growth performance of *Metarhizium anisopliae* and *Beauveria bassiana* isolates under bee colonies' temperatures 60

Abstract 60

4.1. Introduction 61

4.2. Materials and methods 63

4.2.1. Determination of the effect of temperature on conidial germination 63

4.2.2. Determination of the effect of temperature on mycelial growth 64

4.2.3. Nonlinear models for the study 65

4.2.4. Data analysis 67

4.3. Results 68

4.3.1. Comparison of the models for conidial germination ... 68
4.3.2. Comparison of the models for mycelial growth ... 71
4.3.3. Cardinal estimates for conidial germination and mycelial growth ... 74
4.4. Discussion ... 77
Chapter Five ... 81
5.0. Effect of entomopathogenic fungus (*Metarhizium anisopliae*) on survival, pollination behaviour and pollination success of African stingless bee *Meliponula ferruginea* pollinating cucumber *Cucumis sativus* ... 81
Abstract ... 81
5.1. Introduction ... 82
5.2. Material and methods ... 83
5.2.1. Preparation of *Metarhizium anisopliae* icipe 69 ... 83
5.2.2. *Meliponula ferruginea* colony ... 84
5.2.3. Experimental setup ... 84
5.2.4. The application of *Metarhizium anisopliae* icipe 69 ... 87
5.2.5. Determination of flight intensity, foraging activity, and survival of bees ... 87
5.2.6. Determination of fruit set, development, and yield ... 88
5.2.7. Determination of conidial acquisition and pollen load by bees ... 89
5.2.8. Evaluation of persistence and viability of conidia on cucumber crop ... 90
5.2.9. Data analysis ... 90
5.3. Results ... 91
5.3.1. Flight activity, foraging activity, and survival of forager bees ... 91
5.3.2. Fruit set, development, and yield ... 95
5.3.3. Conidial acquisition and persistence ... 97
5.4. Discussion ... 98
Chapter Six ... 102
6.0. General conclusions and recommendations ... 102
6.1. General conclusions ... 102
6.2. Recommendations ... 104
References ... 105

LIST OF FIGURES

Figure

3.1: Micro-spray tower for applying entomopathogenic fungi on filter paper during exposure bioassay and incubators for maintaining exposed bees....................... 46

3.2: Kaplan–Meier survival curves for *Apis mellifera* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*. 50

3.3: Kaplan–Meier survival curves of *Meliponula ferruginea* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*. 51

3.4: Mycosis of caged *Apis mellifera* (**a**) and *Meliponula ferruginea* (**b**) exposed to *Metarhizium anisopliae* and *Beauveria bassiana*... 54

3.5: Scatter plots showing linear relationships between conidial acquisition of *Metarhizium anisopliae*, and *Beauveria bassiana* isolates with LT_{10} (**a**) and mycosis (**b**) for *Apis mellifera*. 55

3.6: Scatter plots showing linear relationships between conidial acquisition of *Metarhizium anisopliae*, and *Beauveria bassiana* isolates with LT10 (a) and mycosis (b) for *Meliponula ferruginea* 56

4.1: Representation of (**a**) conidial viability assessment by enumerating percentage germination of conidia and (**b**) measuring of daily radial growth of the fungus along two cardinal lines intersecting perpendicularly at the bottom centre of Petri dishes. 64

4.2: Curves of nonlinear models predicting the effect of temperature on conidial germination of *Metarhizium anisopliae* and *Beauveria bassiana*. CTMI = cardinal temperature model with inflection.. 69

4.3: Curves of nonlinear models predicting the effect of temperature on mycelial growth of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, and ICIPE 78) and *Beauveria bassiana* (ICIPE 284). CTMI = cardinal temperature model with inflection. 72

5.1: Experimental layout in greenhouses and one greenhouse compartment containing young cucumber *Cucumis sativus* plants.. 85

5.2: Representation of treatment plot containing blooming cucumber *Cucumis sativus* plants and installed with colony of *Meliponula ferruginea*............................... 86

5.3: Stingless bee *Meliponula ferruginea* foraging on a male flower (**a**) and a female flower (**b**) of cucumber *Cucumis sativus*... 88

5.4: Physiological mature cucumber *Cucumis sativus* fruit (14–day–old fruit, tagged with red thread) ready for harvest (**a**) and measurement in the laboratory (**b**). . 89

5.5: Flight activity of *Meliponula ferruginea* in treatment plots containing flowering *Cucumis sativus*. 93

5.6: Foraging activity of *Meliponula ferruginea* in treatment plots containing flowering *Cucumis sativus*. 94

5.7: *Meliponula ferruginea* survival from plots containing treated *Cucumis sativus*.95

5.8: Conidial viability on *Cucumis sativus* after biopesticide spray. 98

LIST OF TABLES

Table

2.1: Potential of pollinators as entomovectors of fungal-based biological control agents (BCAs) in pest management and associated side effects. 31

3.1: Fungal isolates, their origins, target pests and commercialization. 39

3.2: Colony-forming units per bee after exposure to *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78 and *Beauveria bassiana* ICIPE 284.48

3.3: Lethal times (LT) and 95% fiducial limits (FL) for *Apis mellifera* and *Meliponula ferruginea* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*.......... 52

4.1: Comparison of nonlinear models used to predict the effect of temperature on conidial germination of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284................................ 70

4.2: Likelihood ratio test (z values) between the best-fitting model and other models for conidial germination of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *Beauveria bassiana* (ICIPE 284) isolates........... 70

4.3: Comparison of nonlinear models used to predict the effect of temperature on mycelial growth of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284.. 73

4.4: Comparison between the best-fitting model and other models for mycelial growth of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *Beauveria bassiana* (ICIPE 284) isolates. 73

4.5: Models' estimates for conidial germination of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78 and *Beauveria bassiana* ICIPE 284.75

4.6: Models' estimates for mycelial growth for *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78 and *Beauveria bassiana* ICIPE 284.76

5.1: Fruit set and maturation of *Cucumis sativus* pollinated by *Meliponula ferruginea* 9 days before until 9 days after treatment application..................................... 96

5.2: Fruit weight (g) of *Cucumis sativus* pollinated by *Meliponula ferruginea* at 9 days before until 9 days after treatment application. 97

5.3: Biopesticide Colony-forming unitsy (CFU) retained on the *Cucumis sativus* plant, and CFU and pollen collected by *Meliponula ferruginea* foragers. 97

ABBREVIATIONS AND ACRONYMS

Adj. R^2	Adjusted R–squared
AIC	Akaike information criterion
ANOVA	Analysis of variance
API	Africa Pollinator Initiative
APU	Arthropod Pathology Unit
AU–IBAR	African Union–InterAfrican Bureau for Animal Resources
BCA	Biological control agents
BZM	Federal Ministry for Economic Corporation and Development
BOD	Biological oxygen demand
CCD	Colony Collapse Disorder
chi	Chitinase gene
CFU	Colony-forming units
CTMI	Cardinal temperature model
DFID	U.K.'s Department for International Development
DRIP	Dissertation Research Internship Programme.
EPF	Entomopathogenic fungi
Eqn	Equation
FL	Fiducial limit
GAP	Good agricultural practices
GDP	Gross domestic product
icipe	International Centre of Insect Physiology and Ecology
IOBC	International Organization of Biological Control
IUCN	International Union for Conservation of Nature

IPBES	Intergovernmental Science–Policy Platform on Biodiversity and Ecosystem Services
IPM	Integrated pest management
IPPM	Integrated pest and pollinator management
LT	Lethal time-response
PDA	Potato dextrose agar
OATH	Original Australian Trigona Hives
P_{max}	Maximum germination or growth rate
PSI	Pounds per square inch
RH	Relative humidity
rpm	Revolution per minute
SDC	Swiss Agency for Development and Cooperation
Sida	Swedish International Development Cooperation Agency
SDA	Sabouraud dextrose agar
T_{max}	Maximum temperature
T_{min}	Minimum temperature
T_{opt}	Maximum temperature

ABSTRACT

Insect pollination sustains the biodiversity of 90% of wild plants, and 75% of crop species for food and nutritional security. Chemical pesticides used to manage arthropod pests constitute a key driver to the unprecedented declines of insect pollinators worldwide. Hence, biopesticides based on entomopathogenic fungi (EPF) are being promoted as safer alternatives. The effects of EPF on insect pollinators have not been investigated in detail for the application in pollinator-resourced crop systems. Thus, this study screened EPF isolates of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69 and ICIPE 78), and *Beauveria bassiana* (ICIPE 284) for their effect on the Western honey bee (*Apis mellifera*) and African stingless bee (*Meliponula ferruginea*). The study was undertaken at the international centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya, from November 2019 through February 2021. In the first part of the study, groups of 25–30 bees/cage were exposed to surfaces sprayed separately with six isolates (10^8 conidial/mL) or sterile water (control) and incubated for 10 days. The exposure assay was replicated four times and repeated twice for each bee species, and conidial acquisition was evaluated on five bees/cage. *Apis mellifera* acquired more conidia (2.8×10^4–1.3×10^5 colony-forming units [CFU]/bee) than *M. ferruginea* (1.1×10^4–2.3×10^4 CFU/bee) based on the analysis of variance. Except for ICIPE 7, ICIPE 20 and ICIPE 69 which caused significant *A. mellifera* mortality (25.8–40.4%) in the first experiment, none of the isolates had a significant effect on either of the bee species according to survival analysis. The isolates are harmless and/or slightly harmful to bees according to the International Organization of Biological Control classification. Bee colonies inherently thermoregulate their hives and, thus, the second part of the study evaluated the performance of six isolates in bee colonies using eight predictive models describing thermal requirements; (minimum

[T_{min}], optimal [T_{opt}] and maximum [T_{max}] thresholds; and maximal performance [P_{max}]). The isolates were incubated at 12, 16, 20, 24, 28, 32 and 36°C, and conidial germination and mycelial growth were measured and fitted to the models. Models were compared numerically (the Akaike information criterion [AIC], adjusted R^2) and statistically (likelihood ratio test). The best models were the cardinal temperature model with inflection (CTMI) and Ratkowsky 3 for germination; and CTMI, Ratkowsky 2 and Lactin 1 for growth. Temperature nonlinearly affected the isolates' performance and the isolates had different thermal requirements. Germination had T_{min}, T_{opt}, T_{max} and P_{max} of 13.2–14.2°C, 26.2–28.9°C, 35.7–36.3°C and 95.4–100.0%; while growth had 7.0–13.2°C, 25.9–28.4°C, 34.5–37.9°C and 1.36–2.28 mm/day, respectively. The low T_{opt} indicate that the isolates are unlikely to operate in bee colonies. Best-fitting models can be routinely used in the selection and re-evaluation of EPF candidates. The third part of the study involved the application of *M. anisopliae* ICIPE 69 in two greenhouses. Greenhouses were partitioned into plots and planted with cucumber (*Cucumis sativus*) following good agricultural practices. Each plot was installed with a colony of *M. ferruginea* at blooming inception and the crops were sprayed with either ICIPE 69 or sterile water (control). The trials were repeated three times in a completely randomized block design. Colony survival, pollination behaviour, fruit set and yield, and persistence on crops were recorded within 9 days before until 18 days after treatment application. Collected data were analysed using generalized linear models. ICIPE 69 isolate did not result in a significant effect on these parameters while conidial acquisition by foragers and persistence on crops declined periodically. These tiered studies establish that EPF developed in Africa can be safely used in integrated pest and pollinator management (IPPM) programmes.

Keywords: *Apis mellifera*, Entomopathogenic fungi, Nonlinear model, Survivorship.

CHAPTER ONE

1.0. GENERAL INTRODUCTION

1.1. Background to the study

Insect pollination is an essential ecosystem service underpinning 90 and 75% of flowering wild plant and crop species, respectively (IPBES, 2016). This service is universally associated with improving crop yield and quality, a prerequisite to resilient food and nutrition security (Bartomeus *et al.*, 2014; Garibaldi *et al.*, 2013). The global contribution of insect pollination services in commercial crops is currently estimated to be between $267–657 billion USD annually (Porto *et al.*, 2020). Moreover, apiculture and meliponiculture are the upcoming practices especially in Africa (AU–IBAR, 2019). These practices are associated with several hive products such as honey, wax, propolis, bee venom and royal jelly which are increasingly used in several industries including food and medicine industries, forming sources of livelihood to many farmers and stakeholders (Pasupuleti *et al*., 2017). However, there are threats to the global food basket. Notably, most high-commodity and pollinator-dependent crops are under constant attack by arthropod pests. In the context of Africa, the productivity of key crops are decimated by several arthropod pests (Kambura *et al*., 2018; Odanga *et al*., 2017; Sharma *et al*., 2016; Badii *et al*., 2015). As a result, damages and phytosanitary restrictions caused by these pests have prompted heavy applications of chemical pesticides (Badii *et al*., 2015).

Consistent applications of broad-spectrum chemical pesticides have negatively affected nontarget insects (Mullin *et al.*, 2010; Desneux *et al*., 2007). Markedly, chemical pesticides coupled with environmental perturbations and pathogens constitute key stressors to the increasing global declines of pollinators (IPBES, 2016;

Garibaldi *et al*., 2010; Potts *et al*., 2010). Consequently, low crop productivity and dwindling hive product outputs have been documented and are increasingly becoming a global concern threatening food security (IPBES, 2016; Vanbergen *et al*., 2013) and livelihoods of farmers in the crop farming and beekeeping sectors (AU–IBAR, 2019).

Biological control approach based on entomopathogenic fungi (EPF) is considered a better alternative to chemical pest control (De Faria and Wraight, 2007). EPF are increasingly adopted for their eco-friendliness, bio-specificity, and ease of mass production (Maina *et al*., 2018). In Africa, entomopathogenic fungi (EPF), mainly isolates of *Metarhizium anisopliae* (Metsch.) Sorokin, have been developed into biological control products especially during the last two decades (Akutse *et al*., 2020). Their efficacy has been widely demonstrated on several agricultural pests (Niassy *et al*., 2012; Ekesi *et al*., 2007).

During pest mitigation practices in pollinator-based crop systems, EPF applied on crops may affect the survival of forager bees or affect their foraging behaviour including flight activity, flower visitation rate, pollen collection and consequently affecting fruit set and yield of the crops. EPF introduced intentionally or unintentionally into beehives can remain viable, infect the bees and/or contaminate hive products. However, eusocial bees can avoid the effect of EPF through sophisticated hygienic behaviours and inherent thermoregulation of internal hive temperatures to an average range of 31.0–36.0ºC (Jarimi *et al*., 2020). At this temperature range, growth of many EPF is reportedly restricted, however, some can still grow maximally to cause infections (Davidson *et al*., 2003). To expediently describe the temperature-growth interactions of EPF by simulating hive temperatures, suitable predictive models need to be adopted.

Nonlinear models are essential tools widely used in food microbiology to predict the effect of static and dynamic biophysical conditions including temperatures on the growth of food spoilage and toxigenic bacteria (Huang *et al*., 2011; Rosso *et al.*, 1995; Zwietering *et al.*, 1991) and fungi (Peleg and Normand, 2013; Gougouli and Koutsoumanis, 2013, 2012; Dantigny *et al*., 2011). Though, comparatively few temperature-dependent models have been tested on EPF (Davidson *et al.*, 2003; Smits *et al*., 2003; Fargues *et al.*, 1997).

Information on the effect of EPF on bees and their ability to thrive in bee nests are critical to warrant their usage in integrated pest management (IPM) and integrated pest and pollinator management (IPPM) programmes. Thus, the present study evaluated the potential effect of six commercialized EPF isolates of *M. anisopliae* and *Beauveria bassiana* (Bals.) Vuill. on the Western honey bee (*Apis mellifera* L.) (Hymenoptera: Apidae) and the African stingless bee (*Meliponula ferruginea* Cockrell) (Hymenoptera: Apidae) under laboratory and semi-field conditions. These EPF isolates have been registered as Campaign® (*Metarhizium anisopliae* ICIPE 69), Achieve® (*Metarhizium anisopliae* ICIPE 78), Supreme® (*Metarhizium anisopliae* ICIPE 62) and TickOff® (*Metarhizium anisopliae* ICIPE 7) while *Metarhizium anisopliae* ICIPE 20 and *Beauveria bassiana* ICIPE 284 are in pipeline for commercial use (Akutse *et al*., 2020). The effect of bee colonies' conditions on viability and growth of these isolates was assessed using predictive models.

1.2. Statement of the problem and justification of the study

Agriculture is the economic mainstay of many African countries which provides full–time employment to 70% of the population, accounting for one-third of the gross domestic product (GDP) and 40% foreign exchange earnings (AU–IBAR, 2019). In

this sector, pollinators and pests remain insects of economic importance. Pollinators, specifically bees, provide pollination services, hive products and by-products. Therefore, bees need to be conserved to sustainably provide these ecosystem services. On the other hand, arthropod pests decimate crop productivity and pesticides have been broadly used as mitigation measures (Warra and Prasad, 2020), but their application has negatively affected beneficial insects primarily bees (Böhme *et al*., 2018).

EPF as biological control agents (BCAs) are being championed because most of them are arguably harmless to nontarget and beneficial organisms (Zimmermann, 2007) and their residues are unlikely to be traced in agricultural products (Maina *et al*., 2018). Additionally, they are self-perpetuating in the habitats of the pests to provide extended protection and are unlikely to trigger a resistant population of pests compared to chemical insecticides (Kidanu and Hagos, 2020).

However, the ecological risks of currently developed EPF on principal pollinators especially bees remain least explored in detail. Previous laboratory studies have shown variable effects of EPF on bees depending on exposed species of bees (Toledo–Hernandez *et al*., 2016), the species and isolates of EPF (Espinosa–Ortiz *et al*., 2011), and the tested concentrations and exposure methods (Potrich *et al*., 2018). This indicates that candidate EPF may or may not be safe for bees. To explicitly understand the effect of EPF, tiered studies are essentially required. Predictive models, used in forecasting the effect of temperature ranges on growth of several microbes (Peleg and Normand, 2013; Gougouli and Koutsoumanis, 2010) may be important tools in predicting the effect of temperature on EPF in bee colonies. The use of predictive models in describing the growth performance of EPF in conditions of the bee as

pollinators and target pests may be critical in designing IPPM programmes. During the management of pests, EPF applied as biopesticides on flowering crops may impair foraging activities and survival of pollinators and consequently, the reduction of crop yield. Therefore, tiered studies on the nontarget effects of EPF will help in the selection of EPF candidates for the application in pollinator-resourced crop systems.

1.3. Objectives

1.3.1. Broad objective

This study aimed at assessing the nontarget effect of five isolates of *M. anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and one isolate of *B. bassiana* (ICIPE 284) on *A. mellifera* and *M. ferruginea* under laboratory and semi-field conditions, and to predict the germination and growth of these isolates under beehives' temperatures.

1.3.2. Specific objectives

i. To assess the level of conidial acquisition and safety of *M. anisopliae* and *B. bassiana* to *A. mellifera* and *M. ferruginea* under laboratory conditions.

ii. To establish nonlinear models to describe the effect of bee colonies' simulated temperatures on conidial germination and mycelial growth of *M. anisopliae* and *B. bassiana.*

iii. To investigate the effect of *M. anisopliae* on survival, pollination behaviour and pollination efficiency of *M. ferruginea* and establish their persistence on cucumber *Cucumis sativus* L. under greenhouse conditions.

1.4. Research hypothesis

i. *Apis mellifera* and *M. ferruginea* can acquire conidia of biopesticides *M. anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *B. bassiana* (ICIPE 284) but with not any negative affect their survival.

ii. There are predictive models to suitably describe conidial germination and mycelial growth of *M. anisopliae* and *B. bassiana* in hive-simulate temperature

iii. Spraying crops with biopesticides can be retained on crop surfaces but cannot affect *M. ferruginea* survival, pollination behaviour and crop yield.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Pollinators and their ecosystem services

Pollinators are vital in essentially all natural and agricultural ecosystems. Contributions of feral and managed bees in pollination and production of lucrative by-products are unequivocal. Globally, insect pollination services are attributed primarily to honey bees (Garibaldi *et al.*, 2013) and stingless bees (Slaa *et al*., 2006; Herd, 1999). In the context of Africa, bee pollinators comprise the families of Apidae, Halictidae, and Megachilidae (API, 2004). The Western honey bee (*A. mellifera* L.) remains a widely studied bee species because of its relative abundance, pollination services and production of lucrative honey (Muli *et al.*, 2014; Kasina *et al*., 2009; API, 2004). Africa also harbours diverse and abundant species of Afrotropical stingless bees such as *Meliponula* spp., *Hypotrigona Dactylurina* spp., *Plebeina Liotrigona* sand *Cleptotrigona* (Nkoba *et al*., 2012; Eardley, 2004) and their pollination services in different agroecosystems have been widely acknowledged (Asiko, 2012; Kajobe, 2006). Additionally, meliponiculture is upcoming practice due to the production of high-quality honey with medicinal properties that are recognized internationally (Eardley and Kwapong, 2013; Souza *et al*., 2006).

Insect pollinators are prolific actors in crop reproductive success, preconditions for food security, rich plant biodiversity and ecological health (Kumar *et al.*, 2018; Garibaldi *et al.*, 2013). Notably, about 90% of tropical plant species and 78% temperate zone plant species are pollinator-dependent (Potts *et al.*, 2016; Vanbergen *et al*, 2014). The productivity of high-value crops is largely supported by bee pollination and such

crops widely cultivated in Africa include the watermelon (*Citrullus lanatus* Thunberg), pumpkin (*Cucurbita pepo* L., *C. moschata* L., *C. maxima* L.), cucumber (*Cucumis sativus* L.) and avocado (*Persea americana* Miller), mango (*Mangifera indica* L.), passion fruit (*Passiflora edulis* Sims) and eggplant (*Solanum melongena* L.) (Martins, 2014; API, 2004). These crops have almost 65 to 95% insect pollination dependency (Giannini *et al*., 2015; Gallai *et al*., 2009; Klein *et al*., 2007).

Insect pollination is also critical in nutrition security. Potts *et al.* (2016) and Chaplin–Kramer *et al.* (2014) recounted the significant contribution of pollinators in nutrition values of focal crops, a key facet in alleviating malnutrition in many developing countries including Africa. Principal nutrients attributed to insect pollination include lipids, vitamins, iron, folic acid, and minerals (Vanbergen *et al*., 2014; Klein *et al*., 2007).

2.2. Pollination crisis and drivers to the decline of pollinators

Insect pollination is currently considered an endangered ecosystem service at both local and global scale and bees have been listed in the International Union for Conservation of Nature (IUCN) Red List to be under some degree of extinction (IPBES, 2016). The persisting pollinators' declines are linked to ecological perturbations (Potts *et al*., 2010), anthropogenic stressors (Potts *et al*., 2010), pests and diseases (IPBES, 2016; Vanbergen *et al*., 2013; Potts *et al*., 2010) and the use of broad-spectrum chemical pesticides (Ostiguy *et al*., 2019; Kumar *et al*., 2018). Pollinators' declines and consequently declines in pollinator-dependent crops have resulted in widespread economic vulnerability (Potts *et al*., 2010; Biesmeijer *et al*., 2006). Lack or insufficient pollination services on high-commodity and pollinator-dependent crops grown for subsistence and/or commercial purposes have led to their extinction or resulted in low

productivity, hence, elevating economic vulnerability and consequently food insecurity (Biesmeijer *et al*., 2006). Most of the flowering crops (75%) require animal-mediated pollination services, but with variable requirements. Klein *et al.* (2007) sorted the world-leading food crops into six categories based on their reliance on animal-mediated pollination, that is, (1) essential; production require > 90% pollination services; (2) high: production requires 40 to 90%; (3) modest: production requires 10 to 40%; (4) little: production requires 0 to 10%; (5) not required; pollination services cause no production increment; and (6) unknown, for crops with missing data on pollination requirements. A metanalysis by Vanbergen *et al*. (2013) described a global-wise loss of principal pollinators in the last five decades. About 59% and 25% of bee colonies have disappeared in the USA and Europe, respectively (IPBES, 2016; Potts *et al*., 2010). Despite fragmentary data, evidence such as a 40% drop of insect-pollinated orchids in Africa also pinpoints the far-reaching declines (Potts *et al*., 2016).

2.3. Effect of chemical pesticides on pollinators

Chemical pesticides are used to control crop pests and sometimes pollinators' pests like mites (*Varroa* spp.) (Mullin *et al*., 2010). Bees are prototypical insects for assessing the effects of any pesticides on insect pollinators both in laboratory and field conditions. Negative impacts of chemical pesticides on bees have been extensively documented (Henry *et al*., 2012; Mommaerts and Smagghe, 2011b; Mullin *et al*., 2010; Chauzat *et al*., 2009). They have also been traced in bee-collected pollen, honey and bee wax (Ostiguy *et al*., 2019; Wiest *et al*., 2011). Groups of chemical pesticides showing adverse effects on bees include neonicotinoids (Woodcock *et al*., 2017), imidacloprid (Meikle *et al.*, 2018) and organophosphates (Dorneles *et al*., 2017).

Chemical insects have also shown positive aspects in the beekeeping industry by averting Colony Collapse Disorder (CCD) associated with heavy invasion of bee colonies, especially by the *Varroa* spp. and the viruses they transmit (IPBES, 2016). For instance, applications of certain miticides in honey bee colonies have successfully reduced the mite population, however, with a noticeable reduction in honey bee brood area, especially during the spring season (Vandervalk *et al*., 2014). Bahreini *et al.* (2020) tested 16 miticides and found them to be effective in the management of the *Varroa* spp., though for most of the miticides, increasing their doses during application induced bee mortality. Eco-friendly pest management such as using EPF may be considered safer alternatives.

2.4. Entomopathogenic fungi

2.4.1. Overview of entomopathogenic fungi

EPF comprise phylogenetically diverse groups of fungi found naturally in the soil, chiefly parasitizing arthropods (Mora *et al*., 2017). At least twelve classes in kingdom Fungi are insect pathogenic and belong to five divisions *viz.* Zygomycota, Ascomycota, Deuteromycota, Oomycota and Chytridiomycota (Maina *et al*., 2018). Class Hyphomycetes and order Entomophthorales in the divisions Deuteromycota and Zygomycota, respectively, are largely tested and formulated fungi as biological control agents (BCAs) against several arthropod pests. Hyphomycetes such as *B. bassiana* and *M. anisopliae* aggregately account for about 67.8% of marketed fungal-based biopesticides (approx. 171 products) while *Isaria fumosorosea* Wize and *B. brongniartii* Saccardo account for 5.8% and 4.1%, respectively (Maina *et al*., 2018; Sandhu *et al*., 2012). Other commercialised fungal products based on EPF include *Lecanicillium* spp., *Paecilomyces* spp., *Hirsutella thompsonii* Fisher and *Nomuraea*

rileyi Farlow (Sandhu *et al*., 2012). These products have substantially reduced the impact of the arthropod pests of crops, increasing and/or improving the yield in terms of quality and quantity, and consequently the economic return (Kibira *et al.* 2010).

2.4.2. Host selection and mode of action of entomopathogenic fungi

Most EPF infect their host via conidia (also known as spores) with characteristically similar infection processes. Typically these infection processes involve conidial adhesion, germination by the formation of appressorium, penetration of insect cuticle followed by haemocoel and tissue invasion and colonization (Mora *et al*., 2017; Sandhu *et al*., 2012). EPF are pathogenic to nearly all insect orders with Hemiptera, Coleoptera, Lepidoptera, Diptera, Orthoptera and Thysanoptera being more susceptible hosts (Mora *et al*., 2017). Many EPF, chiefly *B. bassiana* and *M. anisopliae* have been reported to infect over 700 insect species, while some EPF such as *Aschersonia aleyrodis* Webber and *N. rileyi* have shown the highest specificity to their targets (Maina *et al*., 2018). In the presence of a susceptible host insect, the infective conidia adhere to its cuticle via non-specific adhesive mechanisms facilitated by the hydrophilic conidia cell-wall proteins like adhesins, which bind strongly to hydrophobic exoskeleton surface and weakly on the hydrophilic surface of the host (Mora *et al*., 2017). Some EPF possess cell-wall moieties like lectins known as hydrophobins, which interact with insect glycoprotein specific receptors to foster a strong adhesion (Mora *et al*., 2017).

Under conducive biochemical (e.g., pH, nutrition) and physical (e.g., temperature, humidity) conditions, the infective conidia produce germination tubes that penetrate the insect cuticle via the synergy of mechanical and enzymatic actions (Mora *et al*., 2017; Shahid *et al*., 2012). The enzymatic actions are performed by cuticle-degrading

enzymes released by conidia including proteases, lipases, chitinases and esterases (Maina *et al*., 2018; Sandhu *et al*., 2012). Some EPF may gain entry into the insect body via spiracles or digestive system (Mora *et al*., 2017). An exposed insect may overcome the effect of the fungus through inhibition of conidial germination and penetration by limiting the required exogenous sources of carbon (e.g. chitin, fatty acids), developing thick and sclerotized epicuticle, production of antifungal compounds (e.g. antimicrobial proteins, phenol oxidases, lectins, peptides) or through cell-mediated response (e.g., phagocytosis, encapsulation) (Mora *et al*., 2017). In susceptible hosts, infections are accelerated by over-expression of genes for proliferation and cuticle-degrading enzymes particularly by *M. anisopliae* and *B. bassiana* (Mora *et al*., 2017).

Invasion of haemocoel and tissues is followed by fungal mycelia undergoing a dimorphic transition to form yeasts or protoplasts (blastospores), which are adaptive stages to avoid recognition by host immune systems and to allow for rapid proliferation (Mora *et al*., 2017). The parasitized insect may display unusual physiological symptoms like low to lack of coordination, altered behavioural responses and paralyses (Mora *et al*., 2017). Death may ensue within 3–14 days after inoculation which may be attributed to tissue destruction, immunosuppression, septicaemia, nutrient deficiency or a combination of these (Maina *et al*., 2018). The insect cadavers always display distinct fungal outgrowth related to the disease-causative fungus including white, green and yellow muscardines for *Beauveria* spp., *Metarhizium* sand *Paecilomyces* spp*.,* respectively (Sandhu *et al*., 2012). Mycelia on mycosed cadavers undergo conidiation and conidia can be dispersed to the next host via contact or wind to initiate the infection cycle again (Sandhu *et al*., 2012).

Toxicity of EPF is also associated with the production of several mycotoxins such as beauvericins, brassinolide and beauverolides produced by both *B. bassiana*, *V. lecanii* and *Paecilomyces* and, destruxins and cytochalasins produced by *M. anisopliae* (Maina *et al*., 2018). These toxins can cause behavioural abnormalities *viz*. general paralysis, sluggishness and reduced irritability due to depolarization of muscle systems, secretion organs and immune systems (Mora *et al*., 2017; Sandhu *et al.*, 2012). Toxins are of interest for ecotoxicological risk assessment in biopesticide regulatory studies.

2.4.3. Entomopathogenic fungi as biological control agents of pests

A plethora of substantial evidence advocates the adoption of microbial BCAs in IPM programmes. Many research and development are focusing on the utilization of EPF as an eco-friendly and soft "option" in IPM strategy (Singh *et al*., 2020; Maina *et al*., 2018) and possibly in IPPM strategy.

Several fungal-based biopesticides have been researched and formulated under different trade names across the world and are being used to control a variety of crop pests in greenhouses, orchards and open fields (Akutse *et al*., 2020; Maina *et al*., 2018). The products comprise of *B. bassiana* (BotaniGard, Naturalis, Betal, Boverol, Ostrinol, Beevicide, Brocaril and Mycontro–WP), *M. anisopliae* (Biologic Bio 1020, Biopath, Bio Blast, Bio Green and Bio Magic), *Lecanicillium lecanii* (Mycota, Verelac and Bioter) and *B. brongniartii* (Betel and Engerlingspilz) (Maina *et al*., 2018; Sandhu *et al*., 2012). In Africa, the most renowned fungal-based biological control products include *M. anisopliae* (e.g. Campaign® [*Metarhizium anisopliae* ICIPE 69], Achieve® [*Metarhizium anisopliae* ICIPE 78], Supreme® *Metarhizium anisopliae* ICIPE 62, TickOff® [*Metarhizium anisopliae* ICIPE 7]) (Akutse *et al*., 2020).

2.5. Application, interaction, and safety of entomopathogenic fungi on bees

2.5.1. Management of crop pests and sensitivity of bees as pollinators

EPF have been successfully used to control numerous crops pests including fruit flies (for example *Ceratitis capitata* Wied., Diptera: Tephritidae; and *Bactrocera invadens* Drew, Diptera: Tephritidae) (Ekesi *et al*., 2007), green peach aphid (*Myzus persicae* Sulzer, 1776, Hemiptera: Aphididae), melon aphid (*Aphis gossypii* Glover, Hemiptera: Aphididae), tobacco whitefly (*Bemisia tabaci* Genn., Homoptera: Aleyrodidae), and greenhouse whitefly (*Trialeurodes vaporariorum* West., Homoptera: Aleyrodidae) a (Sharma *et al*., 2016), flower thrips (*Frankliniella occidentalis* Pergande, Thysanoptera: Thripidae) (Kivett *et al*., 2016) and false codling moth (*Thaumatotibia leucotreta* Meyrick, Lepidoptera: Tortricidae) (Malan *et al*., 2018). However, EPF have been demonstrated to trigger different behavioural reactions of pollinators principally bees, which may include pre-contact avoidance, post-contact avoidance such as grooming, and non–avoidance leading to infection, mortality and transmission (Baverstock *et al*., 2010).

Most EPF pose no adverse effects on bees both in laboratory and field conditions (Challa *et al*., 2019; Soni and Thakur, 2011; Alves *et al.*, 1996). Though some EPF are detrimental to bee survival (Potrich *et al*., 2018; Rodríguez *et al*., 2009), immune response (Hamiduzzaman *et al*., 2012), development (Espinosa–Ortiz *et al.*, 2011), foraging behaviour and nesting recognition (Cappa *et al.*, 2019; Mommaerts *et al*., 2009). However, such effects of the upcoming fungal-based biopesticides are yet to be evaluated on bees.

Laboratory studies by Potrich *et al.* (2018) showed that *B. bassiana* caused a significant reduction in survival of Africanized honey bee (*A. mellifera*) after both oral

and contact treatment compared to *M. anisopliae* treatment. Toledo–Hernandez *et al*. (2016) exposed stingless bees to 1 × 10^8 conidia/mL of different *M*. *anisopliae*, *B. bassiana* and *I. fumosorosea* isolates. The authors found that *M. anisopliae* isolates reduced survival of *Tetragonisca angustula* Latreille, *Melipona beecheii* Bennett and *Scaptotrigona mexicana* Guérin–Méneville by 94.2, 53.0 and 38.9%, respectively. Compared to other bees, species of bumblebees are reportedly less susceptible to EPF (Smagghe *et al.*, 2013; Hokkanen *et al*., 2003). For instance, Smagghe *et al*. (2013) found the bumblebees (*Bombus terrestris* L.) to be only susceptible to 100-fold dose of *M. anisopliae*. However, according to Mommaerts *et al*. (2009), some EPF including registered products based on *B*. *bassiana* have induced significant mortality, reduction in drone production and foraging activity of *B. terrestris*.

The response of bees to EPF at the colony level may be different from that at an individual level. This variation is attributable to colony immunity (Hamiduzzaman *et al*., 2012), hygienic behaviour and hive microclimates such as high temperature (Jones *et al*., 2004). Alves *et al*. (2009) conducted field studies to evaluate the susceptibility of *A. mellifera* (20,000–25,000 bees/hive) by directly exposing them to 1g of each *B. bassiana* and *M. anisopliae*. The authors observed significantly low mortality (< 1%) and no noticeable infections or effects on colony characteristics, behaviour or larval development. However, field studies evaluating the effect of EPF on stingless bees are scarce.

2.5.2. Management of bees' pathogens and safety to bees

Mites (*Varroa destructor* Anderson and Trueman, Mesostigmata: Varroidae), (*V. jacobsoni* Oudemans, Parasitiformes: Varroidae), and (*Acarapis woodi* Rennie, Acari: Tarsonemidae) remain widely disparaging parasites of eusocial bee colonies all over

the world (Potts *et al*., 2010; Klein *et al*., 2007). Numerous EPF have been considered as promising BCAs against these parasites (Celeste *et al*., 2020; Sinia and Guzman–Novoa, 2018; Hamiduzzaman *et al*., 2012). As shown by Meikle *et al.* (2009), *B. bassiana* was aggressive on *V. destructor* and persisted in the hives for more than 50 days to provide extended protection. Kanga *et al*. (2009) showed that *M. anisopliae* induced optimal mortalities to *V. destructor* while causing no significant effect on honey bee colonies. Similarly, *B. bassiana* was effective against *Varroa* mites but induced no significant effects on adult bee survival, brood production or development in the hives (Meikle *et al*., 2008). By contrast, Hamiduzzaman *et al*. (2012) found that *M. anisopliae* and *B. bassiana* affected the immune response of the broods and adult bees even at low concentrations such as 2.62×10^5 conidia/mL.

The small hive beetle (*Aethina tumida* Murray, Coleoptera: Nitidulidae) is an invasive parasitic and scavenging pest of eusocial bees worldwide (Abou–Shaara, 2019) and *M. anisopliae* and *B. bassiana* have been shown as effective BCAs against it (Cuthbertson *et al*., 2013; Muerrle *et al*., 2007). Muerrle *et al.* (2007) recorded more than 30.0% mortality of *A. tumida* after treatment with *B. bassiana* and *M. anisopliae*.

Several EPF have been investigated to be promising in controlling important pests of bees, the greater wax moths (*Galleria mellonella* L., Lepidoptera: Pyralidae) and the lesser wax moths (*Achoria grisella* Fabricius, Lepidoptera: Pyralidae) (Nur *et al*., 2019; Ibrahim *et al.*, 2016). For example, *B. bassiana* and *Paecilomyces lilacinus* Thom have been demonstrated to restrict the *G. mellonella* pupation and adult emergence rates as well as cause optimal larval mortality (> 87.5%) (Ibrahim *et al*., 2016). Likewise, Namusana and Emiru (2010) found that all of the tested six *M. anisopliae* and *B. bassiana* isolates caused < 90% larval mortality to *G. mellonella* but

did not induce significant mortality to the honey bees. Arguably, in these studies, little attention was given to possible lethal and sublethal risks that EPF can pose to bees.

2.5.3. Entomovectoring technology and safety of bees as entomovectors

The advancement in entomovectoring technology largely relies on pollinators as indispensable vectors of fungal-based BCAs (Kevan *et al*., 2008, Al Mazra'awi *et al.*, 2006; Carreck *et al.*, 2006). This is a win-win technology in managing pests of flowering crops, by optimizing the effectiveness of BCAs, reducing chemical pesticides the application and improving pollination efficiency (Kevan *et al.*, 2008). Owing to their adaptive foraging behaviour (e.g. floral constancy), populous colonies and body features such as hairy bodies, bees are renowned to be suitable entomovectors, with special attention given to *A. mellifera*, bumblebees (*Bombus terrestris* L.; and *B. impatiens* Cresson, Hymenoptera: Apidae), and mason bee (*Osmia cornuta* Latreille, Hymenoptera: Megachilidae) (Mommaerts and Smagghe, 2011a; Hokkanen *et al*., 2003). Several studies have shown the potentials of bees as reliable entomovectors (Table 2.1).

The selection of efficient pollinator vectors, effective BCAs, optimal dispensing system and environmental safety is a prevailing challenge in the development of pollinator vector technology (Mommaerts and Smagghe, 2011b; Kevan *et al*., 2008). To warrant the use of bees in this technology, the safety data of BCAs are needed (Carreck *et al*., 2006).

Table 2.1: Potential of pollinators as entomovectors of fungal-based biological control agents (BCAs) in pest management and associated side effects.

Vector	[a]Western honey bee (*Apis mellifera* L.)	[b]Bumblebees (*Bombus impatiens* Cresson)	[c]*A. mellifera.*	[b]*B. impatiens*	[a]*B. terrestris*
BCAs (*CFU/g)	*Metarhizium anisopliae* (5×10^{9})	*Beauveria bassiana* (9×10^{9}, 6.24×10^{10}, 2×10^{11})	*B. bassiana* (1×10^{9})	*B. bassiana* (1.37×10^{10})	*M. anisopliae*
Target crop	Oilseed rape (*Brassica napus* L.)	Tomato (*Solanum lycopersicum* L.), Sweet Pepper (*Capsicum annuum* L.)	*B. napus*	*C. annuum*	–
Target pests	Pollen beetle (*Meligethes aeneus* Fabricius), Cabbage seedpod weevil (*Ceutorhynchus assimilis* Paykull)	Greenhouse whitefly (*Trialeurodes vaporariorum* Westwood), Tarnished plant bug (*Lygus lineolaris* Palisot de Beauvois), Green peach aphid (*Myzus persicae* Sulzer)	*L. lineolaris*	*M. persicae, T. vaporariorum*	–
Side effects on bees	Brood mortality (72.8%), Adult mortality (38.2%)	Adult mortality (43–74%)	–	Adult mortality (25%)	Adult mortality (25–75%)

References: [a] Carreck *et al.* (2006), [b] Kapongo *et al.* (2008), [c] Al Mazra' awi *et al.* (2006), [d] Shipp *et al.* (2012), [e] Smagghe *et al.* (2013). CFU = Colony-forming units.

2.6. Response of bees to entomopathogenic fungi

2.6.1. Immune response against mycotic invasion

Eusocial bees have an elaborate defence mechanism against fungal infections. Whilst bee colony defence is exhibited by resistance such as hygienic behaviour and hypopharyngeal gland secretions, individual bees rely on three essential defences *viz.* protective body coverings, cell-mediated defence such as phagocytosis and encapsulation, and cell-free defence mediated by antimicrobial proteins (Gliñski and Buczek, 2003).

The susceptibility of nearly all insects to EPF is practically age-related, either concomitantly declining or increasing with age. Bull *et al*. (2012) reported that young adult *A. mellifera* nursing bees are less susceptible to EPF due to differential expression of about 35 immune genes compared to foraging bees which express only 2 candidate genes. This suggested that the immunocompetence of bees decreases dramatically at the onset of foraging stages. Hamiduzzan *et al*. (2012) tested *M. anisopliae*, *B. bassiana* and *C. rosea* and found them to be aggressive against *V. destructor* while the inoculated broods of *A. mellifera* resulted in over-expression of protective genes such as *hymenoptaecin*, *pUf68* and *BlCh* which led to low fungal infections of the bees. However, mechanisms contributing to the response of stingless bees to fungal infections are yet to be discovered.

2.6.2. Behavioural responses and protection against fungal invasion

Hygienic behaviour in social insects is one of the heritable genetic mechanisms against pests, pathogens and unusual materials within their proximity. This behaviour has mostly been exhibited by quick detection and removing dead and diseased adults,

larvae and pupae from capped or uncapped brood cells (Al Toufailia *et al.*, 2016; Spivak *et al.*, 2003). Worker bees, through their grooming activity and discarding of debris, can maintain the hygiene of their hives (Gliñski and Buczek, 2003). Stingless bees, especially the Meliponini tribe have high hygienic behaviour. Al Toufailia *et al.* (2016) demonstrated that removal ability of freeze–killed broods after 48 h by stingless bees (*Melipona scutellaris* Latreille), (*Scaptotrigona depilis* Moure), (*Tetragonisca angustula* Latreille) was 99.0%, 80.0% and 62.0%, respectively. In a *A. mellifera* colony, the hygienic activity is performed by 10–15 days old bees nursing bees and this behaviour is mediated by olfactory cues triggered by neuromodulator octopamine that detect infected broods and adults to be removed from the hives (Spivak *et al.*, 2003).

Eusocial bees are super–organisms because of their diverse and sophisticated defence strategies to keep the colony healthy and reproductive. Nesting architecture in stingless bee colonies offers different compartments accessible only to bees with certain functions (Roubik, 2006). Construction materials such as involucres are vital for covering brood combs as well as regulating temperature and ventilation (Roubik, 2006). Additionally, stored materials within the hive such as honey, propolis, bee-collected nectar and pollen contain antimicrobial properties against most fungi and bacteria (Gliñski and Buczek, 2003). However, the behaviour of bees outside the hives especially flight activity and flower visitation rate on crops sprayed with biopesticides is least explored.

2.6.3. Role of bee thermoregulation on mycotic invasion

The intra-hive thermal conditions are complex, which vary with season, hive broodiness (Meikle *et al.*, 2017) and bee species (Jones *et al.*, 2007). For instance,

honey bee colonies typically maintain their brood nests at 32.0–37.0ºC (Meikle *et al*., 2017; Medrzycki *et al*., 2010; Bujok *et al*., 2002). Also, an individual body temperature is crucial in restricting fungal infections. Stabentheiner (2001) observed the thoracic temperatures varied considerably in *A. mellifera* nectar foragers (31.4–43.0ºC) and pollen foragers (37.4–38.0ºC). Both the intra-hive and body temperatures are closely regulated by body metabolism and behavioural responses (Stabentheiner, 2001; Bujok *et al*., 2002).

Coupled with other intra-hive biophysical conditions, hive temperatures directly influence germination, mycelial growth and infectivity of the fungus (Davidson *et al*., 2003). The optimal growth rate of most EPF typically occurs at cardinal temperatures (20.0–30.0ºC). However, a few EPF are tolerant to elevated temperatures > 30.0ºC (Davidson *et al*., 2003) and, therefore, can persist in the hive conditions for an extended period (Kanga *et al*., 2002). Although this persistent may be recommended in managing pests such as *Varroa* spp., EPF may impair the behaviour, activity, development or survival of bees.

CHAPTER THREE

3.0. SAFETY OF *Metarhizium anisopliae* METSCH. AND *Beauveria bassiana* BALS. TO THE HONEY BEE (*Apis mellifera* L.) AND THE STINGLESS BEE (*Meliponula ferruginea* COCKRELL) (HYMENOPTERA: APIDAE) IN LABORATORY CONDITIONS

An article based on this chapter has been published as:

Omuse E. R., Niassy S., Wagacha J. M., Ong'amo G. O., Lattorff H. M. G., Kiatoko K., Mohamed S. A., Subramanian S., Akutse K. S. and Dubois T. (2021). Susceptibility of the Western honey bee *Apis mellifera* and the African stingless bee *Meliponula ferruginea* (Hymenoptera: Apidae) to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Journal of Economic Entomology*. https://doi.org/10.1093/jee/toab211

Abstract

The Western honey bee (*Apis mellifera* L., Hymenoptera: Apidae) and African stingless bee (*Meliponula ferruginea* Cockrell, Hymenoptera: Apidae) are important insects providing pollination services in various agricultural landscapes. They are susceptible to the toxicity of various chemical pesticides. Alternatives such as biopesticides are considered promising in pest management but there is little evidence of their impact on pollinators. The effect of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69 and ICIPE 78) and *Beauveria bassiana* (ICIPE 284) isolates was assessed on *A. mellifera* and *M. ferruginea.* The experiments were conducted at the International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya from November 2019 to July 2020. Young bees were exposed to filter paper sprayed with 1×10^8 conidia/mL alongside a sterile 0.05% Triton-X-100 as a control. Treatments were replicated four times with 25–30 bees per replicate and experiments

were repeated twice. In each replicate, five bees were assessed for conidial acquisition immediately after exposure. *Apis mellifera* picked 2.8×10^4–1.3×10^5 CFU/bee while *M. ferruginea* picked 1.1×10^4–2.3×10^4 CFU/bee. A significant reduction in the *A. mellifera* survival by 40.4, 37.1 and 25.8% was recorded when exposed to ICIPE 20, ICIPE 7 and ICIPE 69, respectively in the first experiment. In the second experiment, *A. mellifera* experienced no fatal effects of the isolates. In both experiments with *M. ferruginea*, none of the isolates caused fatal effects. The significant effects of the isolates on *A mellifera* survival correlated with the number of conidia acquired. The tested isolates are harmless or slightly harmful to bees according to the International Organization of Biological Control (IOBC) classification. However, further studies on the effect of these isolates may be required under field conditions.

3.1. Introduction

The ecological and economic benefits accrued from bee ecosystem services are tremendously declining owing to the indiscriminate application of chemical insecticides (Warra and Prasad, 2020; Brittain *et al*., 2010). Several entomopathogenic fungi (EPF) mainly (*Metarhizium anisopliae* (Metsch.) Sorokin) and (*Beauveria bassiana* (Bals.) Vuillemin) are being endorsed as a substitute to chemical insecticides in most pest management practices worldwide (Akutse *et al*., 2020; De Faria and Wraight, 2007).

Biopesticides based on *M. anisopliae* and *B. bassiana* have been used in sub-Saharan countries. In sub-Saharan Africa, several isolates of biopesticides primarily *M. anisopliae* and *B. bassiana* have been researched for the field application (Akutse *et al*., 2020; Niassy *et al*., 2012; Ekesi *et al*., 2007). EPF isolates; *M. anisopliae* ICIPE 7, *M. anisopliae* ICIPE 62, *M. anisopliae* ICIPE 69, and *M. anisopliae* ICIPE 78 have

been registered as TickOff®, Supreme®, Campaign® and Achieve®, respectively, while *Metarhizium anisopliae* ICIPE 20 and *Beauveria bassiana* ICIPE 284 are in pipeline for commercial use (Akutse *et al*., 2020). These isolates have been researched at the International Centre of Insect Physiology and Ecology (*icipe*) for the management of several pests. The sources, commercial names and target pests of these EPF isolates are presented in Table 3.1.

Although EPF are typically considered target-specific, they have reportedly caused inconsistent effects on bees. For instance, high mortalities caused by some *M. anisopliae* and *B. bassiana* isolates have been recorded on caged *A. mellifera* (Bull *et al*., 2012) as well as on the stingless bees (*Tetragonisca angustula* Latreille, *Melipona beecheii* Bennett and *Scaptotrigona mexicana* Guérin–Méneville, Hymenoptera: Apidae) (Toledo–Hernandez *et al*., 2016). On the other hand, some *M. anisopliae* and *B. bassiana* isolates have caused both high and low mortalities to *A. mellifera* (Espinosa–Ortiz *et al.*, 2011; Al Mazra'awi, 2007) and the stingless bees (*S. mexicana*), (*T. angustula*) and (*M. beecheii*) (Toledo–Hernandez *et al.*, 2016). High mortality caused by EPF may preclude their use as biological control agents (BCAs).

While EPF are being adopted in integrated pest management (IPM) and soon in integrated pest and pollinator management (IPPM) programmes, studies evaluating their safety on bee pollinators are critically essential. Therefore, this study evaluated the effect of the upcoming and commercially available isolates of *M. anisopliae* and *B. bassiana* on *A. mellifera* and *M. ferruginea* under laboratory conditions.

3.2. Materials and Methods

3.2.1. Source and culturing of fungal isolates

This study was conducted at *icipe*, Nairobi, Kenya. Six *M. anisopliae* and *B. bassiana* isolates used in this study, their origin, target pests and commercialization are presented in Table 3.1. Fungal isolates were initially kept in 10% glycerol as slant cultures and preserved at low temperatures (–80ºC).

To revive their virulence, each isolate was inoculated in susceptible host *Galleria mellonella* L. by injecting 7th instar larvae with 5µL sterile water (containing 5,000 conidia). Inoculated larvae were incubated at 25 ± 2ºC in the dark for 7 days. *Metarhizium anisopliae* and *B. bassiana* isolates were cultured on Sabouraud dextrose agar (SDA) (Oxoid, Hampshire, UK) and potato dextrose agar (PDA) (Oxoid), respectively. These media were steam–sterilized for 15 min at 121ºC and 15 PSI in a 63 L autoclave (AMA440, Astell Scientific, Kent, UK), then cooled to 45ºC and 0.25 g/L of an antibacterial agent (streptomycin sulphate) was added followed by dispensing in 95 mm (diameter) × 15 mm (height) plastic Petri dishes. The addition of an antibacterial agent was to restrict contamination of media by other microbes. Conidia from mycosed *G. mellonella* larvae were transferred using a sterile wire loop and streak-plated on media. The processes of adding antibiotics, dispensing the media and inoculating plates with the fungi were performed in a laminar airflow class II biosafety cabinet (SterilCARD, Baker Co., Maine, USA). Inoculated Petri dishes were sealed with parafilm and kept in dark at 25 ± 2ºC for 21 days to ensure maximum sporulation (Dimbi *et al*., 2004).

Table 3.1: Fungal isolates, their origins, target pests and commercialization.

Isolates	ICIPE 7	ICIPE 20	ICIPE 62	ICIPE 69	ICIPE 78	ICIPE 284
Source, Location, Year	Ticks (*Amblyomma* sp.), Rusinga Island (Kenya), 1996	Soil, Migori (Kenya), 1989	Soil, Kinshasa (DR Congo), 1989	Soil, Kinshasa (DR Congo), 1990	Oil palm weevil (*Temnoschoita* sp.), Ungoe	Soil, Unknown (Mauritius), 2005
Current target	Ticks (*Amblyomma* sp., *Rhipicephalus* sp., *Hyalomma* sp.)	–	Aphids (*Aphis craccivorsa* Koch)	Fruit tree mealybug (*Rastrococcus invadens* Williams), *T. frugiperda, L. huidobrensis, Ceratitis* spp., *F. occidentalis*	Two-spotted spider mite (*Tetranynchus urticae* Koch)	–
Trade name	Mazao TickOff®	–	Mazao Supreme®	Campaign®, Real Metarhizium 69®, Real Metarhizium SC®	Achieve®, Mazao Achieve®	–
Future target	Fall armyworm (*Spodoptera frugiperda* Smith), Western flower thrips (*Frankliniella occidentalis* Pergande)	Fruit fly (*Ceratitis* spp.), Tomato leafminer (*Tuta absoluta* Meyrick), Pea leafminer (*Liriomyza huidobrensis* Blanchard), *S. frugiperda*	Fruit fly (*Bactrocera dorsalis* Hendel)	False codling moth (*Thaumatotibia leucotreta* Meyrick), Bean pod borer (*Maruca vitrata* Fabricius)	*S. frugiperda*	*B. dorsalis*, *S. frugiperda*

* Isolates are *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *Beauveria bassiana* (ICIPE 284).
References: Akutse *et al*. (2020), Niassy *et al*. (2012) and Ekesi *et al*. (2007)

3.2.2. Assessment of conidial germination

Germination of the isolates was elucidated by the percentage of germinated conidia before exposure bioassays. Conidia from 21-day old isolate were harvested (Dimbi *et al.* 2004). During harvesting, cultures were flooded with 10mL of sterile 0.05% Triton-X-100 (Triton, Darmstadt, Germany) and the surface of the culture was gently scraped using a sterile soft-tipped spatula. The resulting suspension was transferred to a 25mL universal bottle containing 4 sterile glass beads (1–2 mm diameter). This process was performed in a laminar airflow class II biosafety cabinet. To ensure uniformity, the suspensions were vortexed at 700 rpm for 3 min. Suspensions were serially diluted by transferring 100μL of suspension to 900μL of sterile 0.05% Triton-X-100. The concentration of suspension was established by pipetting and loading droplets of suspension on counting chambers of an improved Neubauer haemocytometer (Marienfeld, Lauda–Königshofen, Germany) followed by counting visible conidia under the light microscope at ×400 magnification. The concentration of suspension was calculated as:

$$Conidia/mL = \frac{Mean\ number\ of\ conidia\ per\ square\ \times\ Dilution\ factor\ \times\ 10^3}{Area\ of\ square\ \times\ Depth\ of\ the\ chamber} \qquad Eqn\ 3.1$$

where the area of a large square is $16 \times 0.0025 = 0.04\ mm^2$ and the depth of the counting chamber is 0.1 mm.

The concentration of suspension of each isolate was adjusted to 3×10^6 conidia/mL by titrating with a sterile 0.05% Triton-X-100. For each isolate, 100μL of the resulting suspension was transferred and spread-plated on fresh media using a sterile L-shaped glass rod. Fresh media was prepared and dispensed in Petri dishes following the

procedure described in section 3.2.1. Fungal suspensions were inoculated in Petri dishes and replicated four times per isolate under a laminar airflow class II biosafety cabinet. Inoculated Petri dishes were sealed with parafilm and incubated in dark at 25 ± 2°C for 18 h. The culture was stained with 2mL lactophenol cotton blue. The stained culture was covered with four glass microscope coverslips (22 × 22 mm) and visible conidia examined microscopically at ×400 magnification. A conidium with a germination tube longer than its width was considered viable. Under each coverslip, exactly 100 conidia, both germinated and non-germinated, were counted, and the percentage of conidial germination was calculated as follows:

$$Conidial\ germination\ (\%) = \frac{Number\ of\ germinated\ conidia}{400\ observed\ conidia} \times 100 \qquad Eqn$$

3.2

3.2.3. Preparation of fungal suspensions

Once conidial germination of above 75% was confirmed, conidia from the remaining Petri dishes were harvested and the concentration of each isolate was determined as described in Section 3.2.2. A constant working concentration of 1×10^8 conidia/mL was prepared for exposure bioassays and maintained at 4ºC for not more than 48 h before being used.

3.2.4. Source and *in vitro* maintenance of *Apis mellifera*

Brood frames of *A. mellifera* were obtained from colonies maintained in standard Langstroth hives at *icipe* apiaries, Nairobi, Kenya (0°13'46"N, 34°51'22"E). The colonies were headed by naturally mated queens and were first established to be healthy using colony strength metrics described by Medrzycki *et al*. (2013) and Delaplane *et al*. (2013). To be selected, a colony was checked for the absence of pests

and pathogens, and for the presence of all developmental stages and castes of bees as well as 710 frames covered with adult bees (1.23–1.77 bees/cm^2), 5 frames with 880 cm^2 containing capped broods and 23 frames containing honey and beebread. Brood frames with mature pupae of worker bees estimated to emerge 1–3 days later were collected from selected colonies. Collected frames were placed in modified wooden cages (30 × 5 × 20 cm) and maintained in complete darkness in 406L high precision biological oxygen-demand (BOD) incubator (MIR–554, PHC Holdings Corporation, Tokyo, Japan) set at 34.5ºC, and 75 ± 5% relative humidity (RH). This RH was achieved using distilled water contained in an open plastic tray (36 × 25 × 3 cm) placed at base compartment of the incubator. These conditions match the condition experienced inside the honey bee central brood areas (Williams *et al.,* 2013).

Every 24 h interval, emerged young bees were gently moved into sleeved Perspex cages (18 × 14 × 14 cm) using a sterile soft camel brush. The first experiment was conducted in November 2019 with 30 bees/cage collected from three selected colonies. The second experiment was conducted in February 2020 with 35 bees/cage collected from the remaining three selected colonies. To minimize possible colony-related effects in experimental outcomes, each cage received an equal number of bees collected from different colonies. Caged bees were provided with 50% (w/v) sugar syrup and 0.5g of pollen collected from *A. mellifera* hives. Sterile Eppendorf tubes were perforated and loaded with the feed and placed in 35 mm (diameter) × 10 mm (height) plastic Petri dishes. To ease cleaning and absorb excess leakage from the feeders, the cages were lined inside with a soft paper towel. Caged bees were allowed to acclimatize to incubation conditions simulating hive conditions of 32ºC and 75 ± 5% RH for one day before the bioassays (Williams *et al.,* 2013).

3.2.5. Source and *in vitro* maintenance of *Meliponula ferruginea*

Colonies of *M. ferruginea* were collected from the *icipe* meliponary at Kakamega forest (0°13'46"N, 34°51'22"E) and transported to *icipe*, Nairobi, Kenya. Colonies were carefully selected by confirming they were free of any pathogens or pests; they have a queen bee and approx. 3,000 adult bees and 7–14 brood combs with essentially all immature developmental stages (eggs, larvae and pupae) and at least 20 storage pots with honey and pollen. Brood combs with old pupae estimated to emerge one week later were collected. Combs were placed in 0.5L perforated plastic cups and maintained in the BOD incubator at *icipe*. To facilitate the emergence of new adults, the combs were maintained in complete darkness and the incubator attuned to 30ºC and 65 ± 5% RH to facilitate the emergency. These conditions simulate the condition experienced inside the stingless bee colonies and are recommended for maintaining stingless bees under artificial conditions (Dorigo *et al*., 2019; Toledo-Hernandez *et al*., 2016).

Newly emerged bees were transferred every 24 h into sleeved Perspex cages using a soft camel brush. The first experiments with *M. ferruginea* were conducted in April 2020 with 30 bee/cage collected from three colonies and repeated in July 2020 with 35 bees/cage collected from the remaining three colonies. Each cage received an equal number of bees from the source colonies. Provision of 70% (v/v) honey-water solution to caged bees were made through perforated Eppendorf tubes positioned in 35 mm (diameter) × 10 mm (height) glass Petri dishes. About 0.5g of beebread collected from *M. ferruginea* colonies were provided as a feed supplement. Caged bees were acclimatized to incubation conditions of 30ºC and 65 ± 5% RH for one day before bioassays as suggested by Williams *et al.,* (2013).

3.2.6. Exposure of caged bees to entomopathogenic fungal isolates

Bees were indirectly exposed to treatments sprayed on surfaces. Treatments consisted of the six isolates alongside sterile 0.05% Triton-X-100 as a control. Sterile water has routinely been used as a control in bioassays evaluating effect of EPF on bees (Colombo *et al*., 2020; Toledo–Hernandez *et al.*, 2016; Conceição *et al*., 2014 ; Bull *et al*., 2012). Whatman filter papers (18 × 14 cm) were sprayed with 10mL of either sterile 0.05% Triton-X-100 or isolate suspension (1×10^8 conidia/mL) using a micro-spray tower (Potter Precision Laboratory Spray Tower, Burkard Manufacturing Co., Hertfordshire, England). Treatments were loaded in tower cuvettes and the tower calibrated to a pressure of 10 PSI. Treatment was applied on filter papers in four replications. Before, between and after any spraying, the tower chambers and cuvettes were surface sterilized with 70% ethanol and rinsed three times with sterile water. Filter papers for the controls were first sprayed followed by the isolate. Filter papers were air-dried for 10 min, introduced at the bottom of the cages and the caged bees were allowed to walk randomly on treated filter papers for 10 min. Treated *A. mellifera* were transferred into Perspex cages while *M. ferruginea* were transferred into 0.5L perforated plastic cups. Cages were lined inside with a clean paper towel.

3.2.7. Assessment of conidial acquisition by the bees

In each experiment, 20 bees per treatment were randomly picked (n = 5) immediately after exposure. To dislodge acquired conidia, bees were individually placed in a universal bottle (10mL) loaded with 1mL of a sterile 0.05% Triton-X-100 and vortexed for 3 min at 700 rpm. Conidia in the resulting suspension were enumerated in counting chambers of Neubauer haemocytometer, and Colony-forming units (CFU)/bee were calculated as follows:

$$CFU/bee = \frac{Mean\ number\ of\ conidia\ per\ square \times Dilution\ factor \times 10^3}{Area\ of\ square \times Depth\ of\ the\ chamber} \qquad Eqn\ 3.3$$

3.2.8. Assessment of the effect of entomopathogenic fungi on bees

The first and second experiments remained with 25 and 30 bees per replicate, respectively and they were *ad libitum* provided with 50% sugar-water solution (*A. mellifera*) and 70% honey-water solution (*M. ferruginea*). The feeds were supplemented with 0.5g beebread collected from the source colonies. BOD incubators were calibrated to 32℃ and 75 ± 5% RH or 30℃ and 65 ± 5% RH for maintaining caged *A. mellifera* and *M. ferruginea,* respectively (Figure 3.1). These conditions match the beehive conditions and are suitable for survival of *A. mellifera* (Williams *et al.* 2013) and *M. ferruginea* ((Dorigo *et al*., 2019) in the laboratory. Bees were always kept in complete darkness except during cleaning, removal of dead bees and replenishing of feed. Daily mortality and survival data were recorded for 10 days post-exposure period.

Only bees confirmed dead were removed from the cages using a pair of forceps. Bees were confirmed to have died when they become motionless by gently touching using a soft camel brush. Dead bees were surface-sterilized in 5% sodium hypochlorite for 1 min and with 70% ethanol for 3 min followed by three times rinsing in sterile water for 1 min. Surface–sterilized cadavers were then individually placed in a 95 mm (diameter) × 15 mm (height) plastic Petri dish lined inside with filter paper moistened with sterile water. Inoculated Petri dishes were sealed with parafilm and incubated in the dark at 25 ± 2℃. Cadavers were checked for mycelial outgrowth within 7 days of post-inoculation. The development of mycosis was an indication that the bee died from a fungal infection.

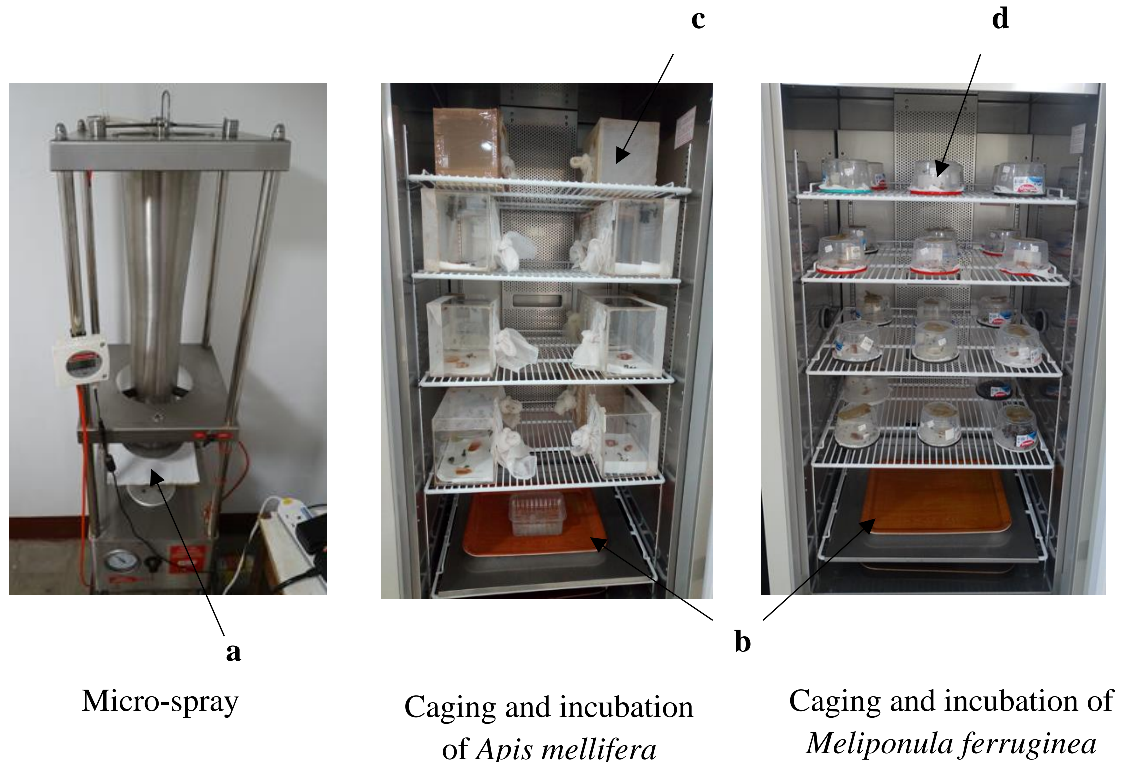


Figure 3.1: Micro-spray tower for applying entomopathogenic fungi on filter paper during exposure bioassay and incubators for maintaining exposed bees.

Note: **a** = filter paper placed on contamination arena of the micro-spray tower, **b** = wooden tray containing distilled water to maintain constant relative humidity, **c** = Perspex cage containing groups of *A. mellifera*, **d** = plastic cage containing groups of *M. ferruginea.*

3.2.9. Data analysis

Data on conidial acquisition by bees were tested for normality (Shapiro–Wilk's test, $p < 0.0001$), log-transformed ($\log_{10}$ [x+1]) and subjected to two–way analysis of variances (two-way ANOVA). Multiple comparisons of means for these data were performed using the *lsmeans* package (Lenth, 2015) with the Tukey method for adjusting p-values.

The *survival* package (Therneau and Lumley, 2020) and the *survminer* package (Kassambara *et al.* 2020) were used to analyse the post-exposure survival of bees. To assess the statistical differences between experiments, among treatments and their

interactions, the Cox proportion hazard model was deployed. Survival distribution curves were generated using the Kaplan–Meier estimator and statistical differences between survival curves were assessed using the log-rank test (also called Cox–Mantel test). The *pairwise_survdiff* function was used for pairwise comparison of means and the Bonferroni p-value adjustment method was used to reduce chances of type I error. The *multicompView* package (Graves *et al*., 2019) was used to summarize the differences in survival curves by generating significant letters.

Daily percentage mortality was corrected using Abbott's correction formula (Abbott, 1925) to eliminate the natural mortality as follows:

$$Adjusted\ daily\ mortality\ (\%) = \frac{Trt - Ca}{100 - Ca} \qquad Eqn\ 3.4$$

where *Ca* and *Trt* are the daily control mortality and daily treatment mortality, respectively. Corrected mortality was subjected to probit regression analysis in the *ecotox* package (Hlina, 2020). This analysis gave the values for lethal time-response mortality to 10% (LT_{10}) and 25% (LT_{25}) of the population and corresponding 95% fiducial limits (FLs). Significant differences in LT_{10} and LT_{25} among treatments was assessed based on the degree of overlaps in their 95% FL.

Mycosis dataset was subjected to logistic regression and mean separation performed using the *glht* function in a *multicomp* package (Hothorn *et al*., 2008) with the Tukey test. The Pearson product-moment correlation was used to assess the linear relationships between conidia acquisition with the LT_{10} and mycosis. All data were analysed using R software (R Core Team, 2020).

3.3. Results

3.3.1. Conidial acquisition by bees

Bees acquired conidia when exposed to surfaces sprayed with suspensions of the six isolates. Significant differences in conidial acquisition were evident between bee species ($p < 0.0001$), among isolates ($p < 0.0001$) and species-isolates interactions ($p < 0.0008$). *Apis mellifera* acquired significantly highest conidia when exposed to ICIPE 20 followed by ICIPE 7, ICIPE 62 and ICIPE 78 and the least when exposed to ICIPE 284. Conidial acquisition by *M. ferruginea* did not differ significantly among the isolates ($p = 0.096$) (Table 3.2).

Table 3.2: Colony-forming units per bee after exposure to *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78 and *Beauveria bassiana* ICIPE 284.

Isolate	***Apis mellifera***	***Meliponula ferruginea***
	Mean (± SE × 10^4)	**Mean (± SE × 10^4)**
ICIPE 7	8.03 ± 0.01b	1.85 ± 0.01a
ICIPE 20	12.97 ± 0.06c	2.11 ± 0.02a
ICIPE 62	9.49 ± 0.03b	2.00 ± 0.03a
ICIPE 69	7.03 ± 0.01b	2.28 ± 0.03a
ICIPE 78	7.25 ± 0.03b	1.90 ± 0.01a
ICIPE 284	2.83 ± 0.05a	1.14 ± 0.01a
p-value	< 0.0001	0.096

Same small letters within the columns indicate no significant differences at $\alpha = 0.05$ according to the Tukey test. SE = standard error.

3.3.2. Post-exposure survival of bees

Over 53.0% of *A. mellifera* and 81.8% of *M. ferruginea* remained alive 10 days post-exposure. For *A. mellifera*, survival was significantly affected by experiments ($p = 0.0016$) and treatments ($p < 0.0001$), but not by their interactions ($p = 0.39$). Significant differences in survival of *A. mellifera* were detected in the first experiment ($p < 0.0001$)

and not in the second experiment (p = 0.061). In the first experiment, survival of *A. mellifera* after exposure to isolates ICIPE 20 (53.0%), ICIPE 7 (56.0%) and ICIPE 69 (66.0%) were significantly different from the control (89.0%) (Figure 3.2).

There were no significant differences in survival of *M. ferruginea* between experiments (p = 0.70), among treatments (p = 0.37) or their interactions (p = 0.91). Survival of *M. ferruginea* after exposure to fungal isolates did not differ significantly from the control (Figure 3.3). Generally, the controls had the highest survival in both bioassays with *A. mellifera* (> 84.2%) and *M. ferruginea* at (> 89.0%).

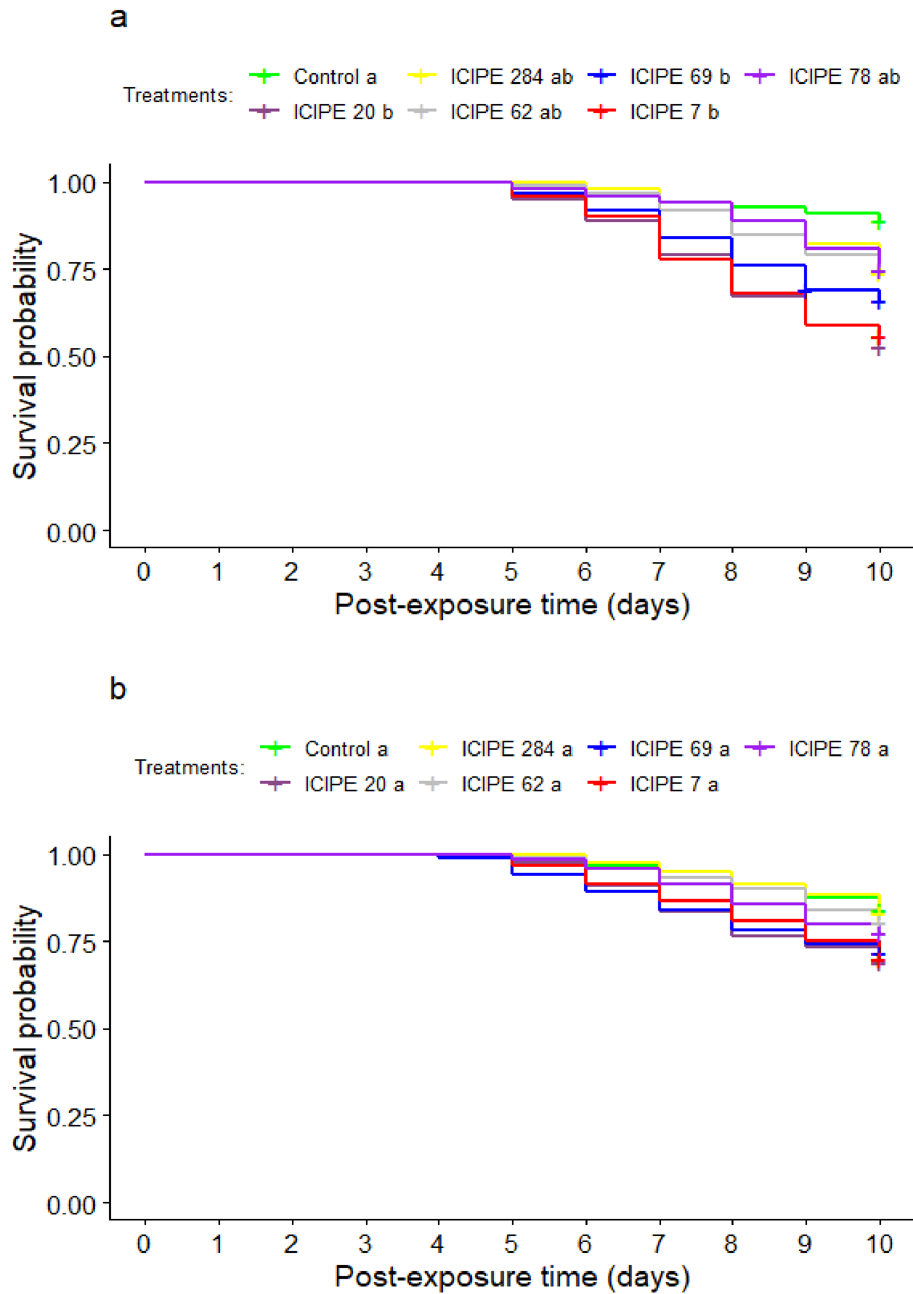


Figure 3.2: Kaplan–Meier survival curves for *Apis mellifera* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*.

Note: Same small letters adjacent to the legends indicate no significant difference in survival distribution curves at $p > 0.05$ according to the log-rank test and Bonferroni p-value adjustment. **a:** First experiment, N = 100 bees/treatment. **b:** Second experiment, N = 120 bees/treatment. "+" indicates right censorship.

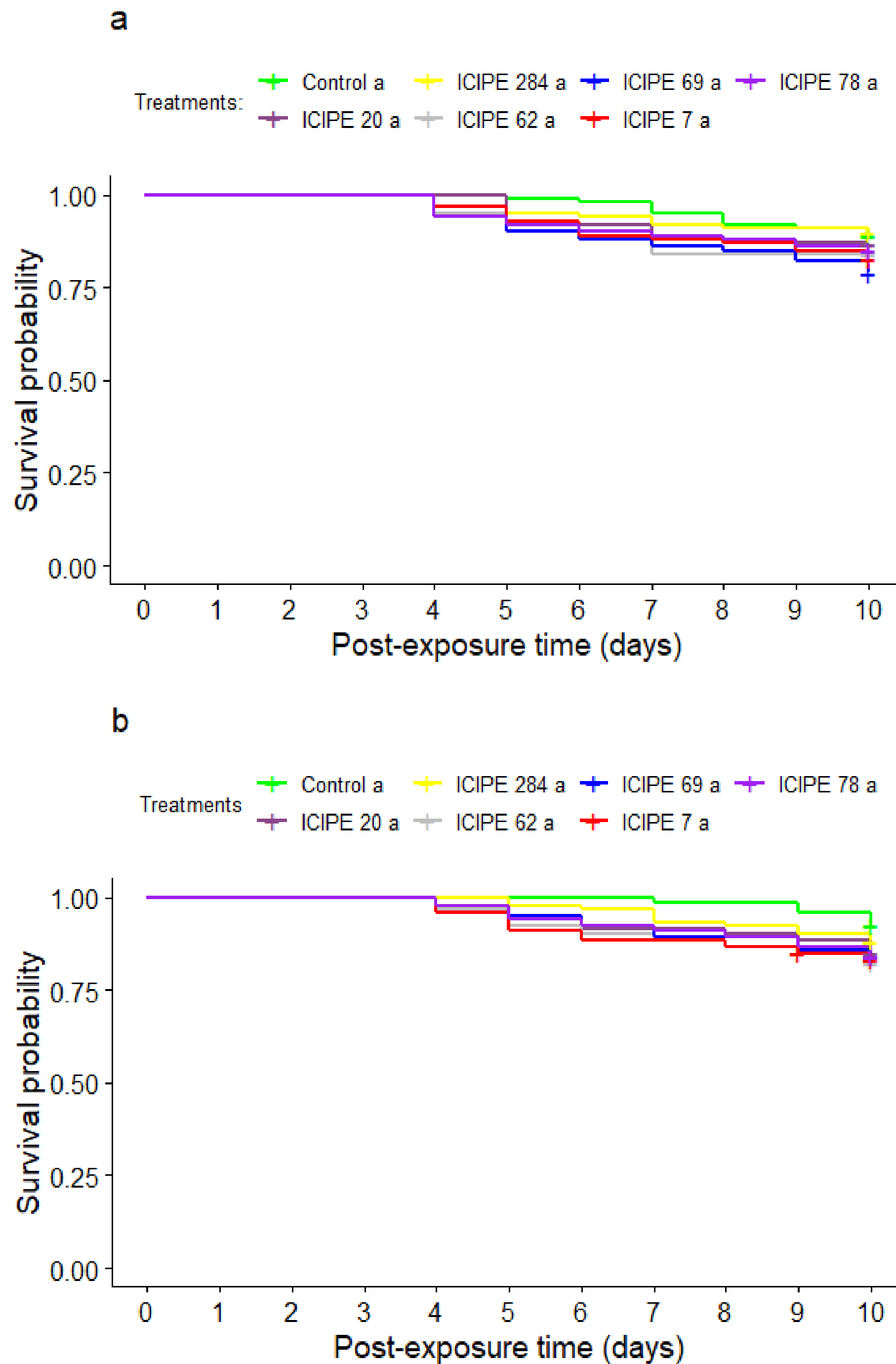


Figure 3.3: Kaplan–Meier survival curves of *Meliponula ferruginea* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*.

Note: Same small letters adjacent to the legends indicate no significant difference in survival distribution curves at $p > 0.05$ according to the log-rank test and Bonferroni p-value adjustment. **a:** First experiment, N = 100 bees/treatment. **b:** Second experiment, N = 120 bees/treatment. "+" indicates right censorship.

3.3.3. Time-response **mortality of fungus-exposed bees**

In this study, treatment mortality of bees did not exceed 39.8% while control mortality did not exceed 15.0%, therefore, LT_{10} and LT_{25} were estimated (Table 3.3). In the first experiment, LT_{10} and LT_{25} for *A. mellifera* were both shorter in treatments with ICIPE 7, ICIPE 20 and ICIPE 69 than in treatments with ICIPE 62, ICIPE 78 and ICIPE 284. In the second experiment, LT_{10} for *A. mellifera* were the shortest in treatments with ICIPE 62 followed by ICIPE 20 and ICIPE 69 treatments, longer in treatment with ICIPE 7, and longest in treatments with ICIPE 78 and ICIPE 284, while LT_{25} were the shortest in treatments with ICIPE 20 followed by ICIPE 7, ICIPE 62, ICIPE 78 and ICIPE 284 treatments, and longest in treatments with ICIPE 78 (Table 3.3).

Table 3.3: Lethal times (LT) and 95% fiducial limits (FL) for *Apis mellifera* and *Meliponula ferruginea* exposed to *Metarhizium anisopliae* and *Beauveria bassiana*.

Trial	*Apis mellifera*		*Meliponula ferruginea*	
	LT_{10}	LT_{25}	LT_{10}	LT_{25}
First experiment				
ICIPE 7	6.1 (5.7, 6.5)a	8.0 (7.6, 8.4)a	8.2 (7.0, 10.2)abc	16.0 (12.1, 28.5)ab
ICIPE 20	6.1 (5.7, 6.4)a	7.9 (7.6, 8.4)a	9.3 (7.5, 15.6)c	17.6 (12.0, 69.6)ab
ICIPE 62	8.4 (7.9, 8.8)c	11.2 (10.4, 12.6)c	7.2 (5.8, 9.8)a	15.3 (10.9, 39.1)ab
ICIPE 69	6.8 (6.3, 7.2)ab	9.2(8.7, 9.9)b	6.5 (5.6, 9.8)a	11.9 (9.9, 16.6)a
ICIPE 78	8.8 (8.3, 9.4)c	12.3 (11.2, 14.2)c	8.8 (6.9, 14.0)bc	20.4 (13.2, 76.3)ab
ICIPE 284	8.9 (8.7, 9.1)c	11.2 (10.8, 11.7)c	10.2 (9.4, 15.5)cd	42.0 (19.5, 76.5)b
Second experiment				
ICIPE 7	8.0 (7.7, 8.4)bc	11.3 (10.6, 12.4)c	6.5 (5.7, 7.3)a	11.4 (9.8, 14.5)a
ICIPE 20	7.5 (7.1, 7.9)abc	10.4 (9.7, 11.4)bc	10.6 (9.0, 14.1)cd	21.4 (15.5, 40.9)ab
ICIPE 62	6.5 (5.7, 7.4)a	11.7 (9.9, 15.6)c	7.1 (6.4, 8.1)a	12.9 (10.8, 17.6)a
ICIPE 69	7.3 (6.7, 7.8)ab	10.7 (9.7, 12.3)bc	7.3 (6.8, 7.8)a	11.9 (10.7, 13.8)a
ICIPE 78	9.6 (9.0, 10.5)d	12.8 (11.5, 15.5)c	8.0 (7.4, 8.8)ab	13.9 (12.0, 17.4)a
ICIPE 284	10.6 (10.3, 10.9)d	11.7 (11.3, 12.3)c	9.5 (8.8, 10.6)c	14.7 (12.7, 36.5)ab

LT_{10} and LT_{25} are lethal time-response mortality to 10% and 25% of the population. Values provided in the brackets are the upper and lower 95% fiducial limits. Same small letters within the columns indicate no significant differences in LT_{10} or LT_{25} at $\alpha = 0.05$ as indicated by overlapping 95% FL.

For *M. ferruginea*, LT_{10} were not significantly different among treatments in the first experiment but LT_{25} were the shortest in treatments with ICIPE 69 followed by ICIPE

7, ICIPE 20, ICIPE 62 and ICIPE 78, and longest with ICIPE 284 in the first experiment (Table 3.3). In the second experiment with *M. ferruginea*, LT_{10} were the shortest in treatments with ICIPE 7 followed by ICIPE 62 and ICIPE 69, longer in treatment with ICIPE 78, and longest with ICIPE 20 and ICIPE 284, while LT_{25} were shortest in treatments with ICIPE 7 followed by ICIPE 62, ICIPE 69, ICIPE 78 and ICIPE 284, and longest with ICIPE 20 (Table 3.3).

3.3.4. Mycosis of fungus-exposed bees

Cadavers from the controls had no detectable mycosis in both experiments with *A. mellifera* and *M. ferruginea*. The percentage of exposed *A. mellifera* and *M. ferruginea* developing mycosis did not exceed 18.5% and 8.0%, respectively (Figure 3.4). No significant difference in mycosis was evident between experiments for *A. mellifera* ($p = 0.45$) and *M. ferruginea* ($p = 0.26$). Though, significant effects were detected in mycosis for *A. mellifera* among isolates ($p < 0.0001$) with ICIPE 7 and ICIPE 20 having the highest mycosis followed by ICIPE 69. However, the lowest mycosis of *A. mellifera* was observed in treatments with ICIPE 62, ICIPE 78, and ICIPE 284. In experiments with *M. ferruginea*, significant differences in mycosis were detected among isolates ($p = 0.0017$), with ICIPE 7, ICIPE 62 and ICIPE 69 exhibiting the

highest mycosis, and ICIPE 20 and ICIPE 78 exhibiting the lowest mycosis. None of *M. ferruginea* treated with *B. bassiana* had mycosis.

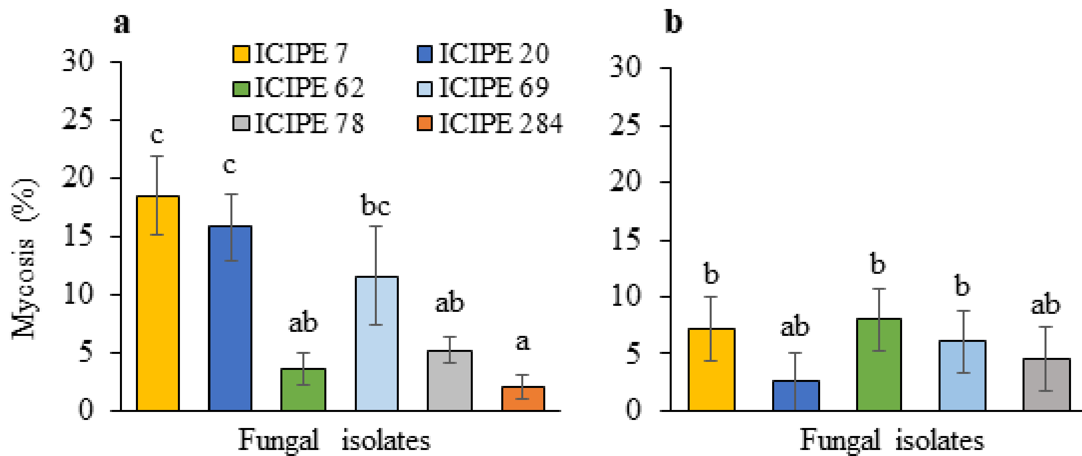


Figure 3.4: Mycosis of caged *Apis mellifera* (**a**) and *Meliponula ferruginea* (**b**) exposed to *Metarhizium anisopliae* and *Beauveria bassiana*.

Note: Error bars represent standard errors. Same small letters above the error bars for each bee species indicate no significant differences in mycosis ($p < 0.05$) based on logistic regression and the Tukey p-value adjustment method. ICIPE 284 had no mycosis on *M. ferruginea*.

3.3.5. Correlation of conidial acquisition with pathogenicity of fungi

A strong negative correlation of conidial acquisition with LT_{10} was evident when *A. mellifera* was exposed to ICIPE 69 ($R = -0.84$, $p = 0.009$), while weak when *A. mellifera* was exposed to ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 78, and ICIPE 284 (Figure 3.5a). Positive correlations were observed between conidial acquisition with mycosis which was strong when *A. mellifera* was exposed to ICIPE 7 ($R = 0.89$, $p = 0.003$) and ICIPE 20 ($R = 0.84$, $p = 0.003$), and weak when *A. mellifera* was exposed to ICIPE 69. ICIPE 62, ICIPE 78 and ICIPE 284 (Figure 3.5b).

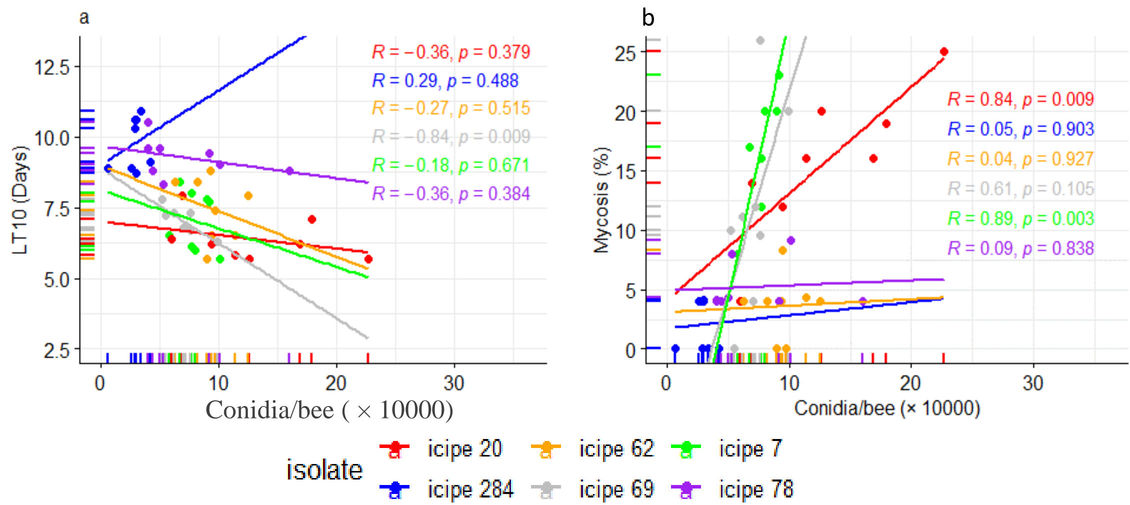


Figure 3.5: Scatter plots showing linear relationship between conidial acquisition of *Metarhizium anisopliae*, and *Beauveria bassiana* isolates with LT_{10} (**a**) and mycosis (**b**) for *Apis mellifera*.

In experiments with *M. ferruginea*, the positive correlation of conidia acquisition was only significant with mycosis caused by ICIPE 69 (R = 0.78, p = 0.023). However, no significant correlation with either LT_{10} or mycosis was confirmed in ICIPE 7, ICIPE 20, ICIPE 62, and ICIPE 78 treatments (Figure 3.6a). None of *M. ferruginea* treated with ICIPE 284 had mycosis and therefore, there was no correlation between conidial acquisition and mycosis (Figure 3.6b)

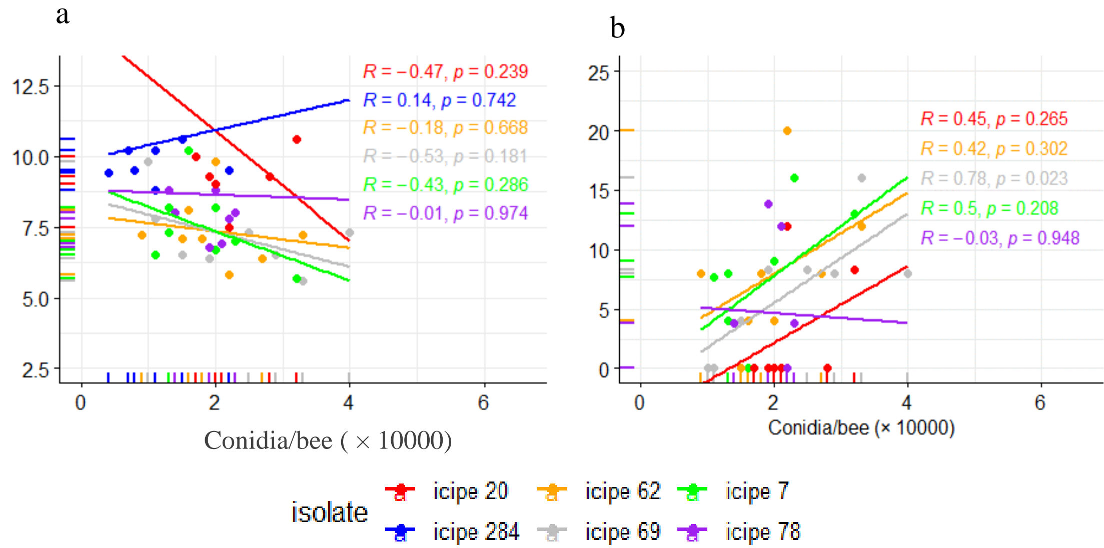


Figure 3.6: Scatter plots showing linear relationship between conidial acquisition of *Metarhizium anisopliae*, and *Beauveria bassiana* isolates with LT_{10} (**a**) and mycosis (**b**) for *Meliponula ferruginea*.

3.4. Discussion

In this study, *A. mellifera* and *M. ferruginea* picked conidia (1.1×10^4–1.3×10^5 CFU/bee) when exposed to a concentration of 1×10^8 conidia/mL of the *M. anisopliae* and *B. bassiana* isolates for 10 mins. However, more conidia were picked by *A. mellifera* than *M. ferruginea* and this difference can be explained by their differential behavioural and morphological traits. With no direct reference comparing the two tested bee species, the body features such as facial area and hairiness of several pollinators have been associated with the number of pollen collected by the bee species (Goulnik *et al*., 2020). *Apis mellifera* has relatively larger (total body length of 14.4 mm) (Adeoye *et al*., 2020) than *M. ferruginea* (total body length of 7.5 mm) (Eardley, 2004) and, therefore, the body size may be one of the features that directly correlate with the level of conidia acquired by each bee species.

Unlike for *M. ferruginea*, the number of conidia acquired by *A. mellifera* differed significantly among the isolates. This could be due to differences in conidial size, hydrophobicity, surface adhesive chemicals such as adhesins in *Metarhizium* species (Mora *et al*., 2017; Liu *et al.*, 2003), and Lectin-binding proteins in *Beauveria* species (Wanchoo *et al*., 2009). *Metarhizium* species have large conidia (8.5 µm length and 2.8 µm width) while *Beauveria* species have relatively smaller conidia (2.1–2.6 µm diameter) (Liu *et al*., 2003). Additionally, differences in conidial acquisition between the bee species can be related to the chemical composition of the host insect cuticle including long-chain hydrocarbons which are known to be fungus-specific (Greenfield *et al*., 2014). Cuticular hydrocarbons that are specific to tested isolates may be lacking on *M. ferruginea*.

Survival of *A. mellifera* was higher in the second experiment than in the first experiment while survival of *M. ferruginea* remained similar in both experiments. A noticeable effect of EPF on *A. mellifera* survival may be effectuated by intrinsic colony strength at the time of sampling, small sample size, or laboratory conditions. Reportedly, maintaining small groups of *A. mellifera* in laboratory conditions makes them more stressful and susceptible to *B. bassiana* and *M. anisopliae* (Al Mazra'awi, 2007; Alves *et al*., 1996). Additionally, the bees lacked the allogrooming behaviour that is normally exhibited in their natural settings, and this may have led to low survival rates.

In this study, corrected bee mortality did not exceed 40.4%. Only *A. mellifera* exposed to ICIPE 7, ICIPE 20, and ICIPE 69 experienced a significant reduction in survival by 25.8–40.4% in the first experiment while the remaining isolates exhibited no significant effect on *A. mellifera* survival. These findings concur with laboratory

findings by Espinosa–Ortiz *et al*. (2011) where caged *A. mellifera* experienced < 12.7% mortality when treated with certain isolates of *M. anisopliae* and *B. bassiana* in 10–day bioassays. Butt *et al*. (1994) also observed mortality of between 29–35% of *A. mellifera* exposed two virulent isolates of *M. anisopliae* at low concertation (1×10^7 conidia/mL), however, > 94.0% mortality with short LT_{50} (4.4–8.5 days) and 100% mycosis were recorded when the concentration was raised to 1×10^{10} conidia/mL in 14-day bioassays. A laboratory study by Colombo *et al*. (2020) demonstrated a reduction in survival of *A. mellifera* after 6 days of treatment with *M. anisopliae* (12.5%) and *B. bassiana* (50.0%). These observations justify that tested isolates can be harmless to *A. mellifera*.

The effect of EPF on the African stingless bees especially *M. ferruginea* has never been reported. In this study, the survival of *M. ferruginea* was not affected by the isolates. However, previous reports on the Neotropical stingless bee such as *Tetragonisca angustula*, *Melipona beecheii* and *Scaptotrigona mexicana* (Toledo–Hernandez *et al*., 2016) as well as *Melipona scutellaris* (Conceição *et al*., 2014) have indicated that *B. bassiana* and *M. anisopliae* have low effect survival on the survival of these bees.

The tested isolates are very aggressive to arthropod pests occurring in the order; Diptera, Hemiptera, Lepidoptera and Arachnida (Akutse *et al*., 2020), however, they caused significantly low (< 18.5%) infection cases on bees as recorded in this study. This suggests that the bees are probably the nontarget insect group or could have developed resistance against these isolates. Bull *et al*. (2012) and Hamiduzzaman *et al*. (2012) demonstrated that the low effect of *M. anisopliae* and *B. bassiana* to *A.*

mellifera could be related to the increased expression of immune-related genes such as *abaecin*, *defensin–2* and *hymenoptaecin*.

Mycosis and LT_{10} of *A. mellifera* after exposure to ICIPE 7, ICIPE 20 and ICIPE 69 correlated strongly with the number of acquired conidia. For *M. ferruginea,* exposure to ICIPE 69 resulted in a strong correlation between the number of acquired conidia with mycosis. The pathogenicity of these isolates can be ascribed to genetics and target pests (Akutse *et al*., 2020; Gao *et al*., 2020). Explicitly, ICIPE 7, ICIPE 20 and ICIPE 69 which have shown some effects on bees, have been reported by Niassy *et al.* (2013) to possess strong correlated performance on diverse pest groups and this may be associated with the chitinase *chi2* and *chi4* genes in addition to genes for toxin production and conidiation.

This study finds *A. mellifera* to be more vulnerable to the effect of *M. anisopliae* than *M. ferruginea*. This difference can also be related to the number of conidia acquired by the bee species and/or differential ant-fungal immunity of these species. Compared to the *B. bassiana* isolate, the effects of *M. anisopliae* ICIPE 7, ICIPE 20 and ICIPE 69 on bees were more profound on *A. mellifera*. Apart from their virulence genes such as subtilisin-like *Pr1* genes (Gao *et al*., 2020), the effects of *M. anisopliae* can be related to faster conidial germination and mycelial growth compared to *B. bassiana*, traits that may be genetic (Liu *et al*., 2003).

This study endorses the application of the tested isolates in IPPM; however, the effect of isolates ICIPE 7, ICPE 20 and ICPE 69 on bees may need to be evaluated under field conditions to warrant their safety as biological control agents. Besides, the effect of elevated temperatures of the hives is needed to forecast the ability of EPF to remain viable and grow under the hive conditions.

CHAPTER FOUR

4.0. MODELLING GROWTH PERFORMANCE OF *Metarhizium anisopliae* AND *Beauveria bassiana* ISOLATES UNDER BEE COLONIES' TEMPERATURES

An article based on this chapter has been published as:

Omuse E. R., Niassy S., Wagacha J. M., Ong'amo G. O., Azrag A. G. A. and Dubois T. (2021). Suitable models to describe the effect of temperature on conidial germination and mycelial growth of *Metarhizium anisopliae* and *Beauveria bassiana*. *Biocontrol Science and Technology*. https://doi.org/10.1080/09583157.2021.1993133

Abstract

Entomopathogenic fungi (EPF) may unintentionally be introduced into beehives by a bee after foraging on treated crops. Bees thermoregulate their hive to average optima of 31–36°C and these temperatures may affect the performance of EPF. The study to assess this effect was conducted at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. Six *Metarhizium anisopliae* and *Beauveria bassiana* isolates were inoculated on selective Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA), respectively, and their germination and growth were recorded at 12, 16, 20, 24, 28, 32 and 36°C. Eight predictive models were used to estimate the cardinal minimum (T_{min}), optimal (T_{opt}) and maximum (T_{max}) thresholds; and maximal germination or growth (P_{max}). The Akaike information criterion (AIC) and adjusted R^2 were used to assess models' goodness-of-fit and best-fitting models selected based on the likelihood ratio test. Temperature had a nonlinear effect on the performance of EPF. The isolates had T_{min}, T_{opt}, T_{max}, P_{max} estimates for germination of 13.2–14.2°C,

26.2–28.9°C, 35.7–36.3°C and 94.4–100.0%, respectively, while the T_{min}, T_{opt}, T_{max}, P_{max} for growth was 7.0–13.2°C, 25.9–28.4°C, 34.5–37.9°C and 1.36–2.28 mm/day, respectively. The generalized β function poorly fitted germination and growth datasets. Brière 1, Brière 2, Ratkowsky 2, Lactin 1 and Van Der Heide models poorly fitted most germination and growth datasets. Cardinal temperature model with inflection (CTMI) and Ratkowsky 3 suitably described all germination and growth datasets while Ratkowsky 2, and Lactin 1 models suitably described some growth datasets. T_{opt} estimates of isolates were below the temperature encountered in central bee brood areas and therefore, they are unlikely to germinate and grow optimally in bee colonies.

4.1. Introduction

Temperature is a key limiting factor to the initial stages of the fungal infection process including germination and mycelial growth (Dimbi *et al*., 2004). Most EPF are capable of growth at diverse temperatures (Rangel *et al*., 2005; Davidson *et al*., 2003; Fargues *et al*., 1997). Of concern are EPF that can remain viable and grow optimally at temperature conditions found in bee colonies (Miętkiewski *et al*., 2014). Several species of honey bees and stingless bees maintain their internal nesting temperatures as high as 32.0–36.0ºC at central brood areas (Jarimi *et al*., 2020) and 31.0–32.3ºC in involucres (Jones and Oldroyd, 2006), respectively. While the optimal germination and growth of most EPF occur at 20–30ºC, some EPF can operate at elevated temperatures matching those of the beehives (Davidson *et al*., 2003). As part of the safety evaluation and selection of EPF for usage in bee-pollinated crop systems, predictive models may accurately describe the performance of EPF in the conditions of the bees.

Numerous mathematical models with varying complexity have been used to describe the influence of biophysical conditions especially temperature on microbial growth. These nonlinear models have been extensively used in food microbiology to forecast the growth of spoilage bacteria (Huang *et al*., 2011; Rosso *et al*., 1995; Zwietering, *et al*., 1991) as well as spoilage and mycotoxigenic fungi (Peleg and Normand, 2013; Gougouli and Koutsoumanis, 2013, 2012; Dantigny *et al*., 2011). These models include the CTMI originally developed by Rosso *et al* (1995), the square root Ratkowsky 2 (Ratkowsky *et al*., 1983), and the modified Ratkowsky 3 (Zwietering *et al.*, 1991). Models Lactin 1 (Lactin *et al*., 1995*)*, Brière 1, and Brière 2 (Briere *et al*., 1999) were originally developed to model the development of insects while the Van Der Heide model was developed by Van Der Heide *et al*. (2006) to predict the effect of temperature on the growth of free-floating macrophytes. The generalized β function was modified by Bassanezi *et al*. (1998) to describe the effect of temperature on the monocyclic components of the bean rust *Uromyces appendiculatus* Persoon and angular leaf spot *Phaeoisariopsis griseola* (Sacc.) Ferraris infecting bean *Phaseolus vulgaris* L. cultivars.

Comparatively, there are few predictive models tested on EPF (Davidson *et al*., 2003; Smits *et al*., 2003; Fargues *et al*., 1997). Models such as Brière 1, Brière 2 and Ratkowsky 3 have previously been used to model mycelial growth of the EPF *M. anisopliae*, *B. bassiana* and *Paecilomyces fumosoroseus* as a function of temperature (Smits *et al*., 2003) while the generalized β function has been used to model the effect of temperature on mycelial growth of various EPF *M. anisopliae* and *B. bassiana* isolates (García–Fernández *et al*., 2008; Quesada–Moraga *et al*., 2006). Most of these models have expediently predicted the effect of temperature on tested EPF. Therefore, this study aimed to validate eight models include those yet to be tested on EPF and to

establish suitable models for describing and predicting the growth performance of promising EPF in hive conditions. Additionally, EPF isolates were compared based on their cardinal thermal requirements.

4.2. Materials and Methods

4.2.1. Determination of the effect of temperature on conidial germination

This study was undertaken at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. The effect of temperature on conidial germination of *M. anisopliae* and *B. bassiana* isolates presented in Table 3.1 in Chapter three was evaluated. These isolates were cultured on SDA (for *M. anisopliae*) or PDA (for *B. bassiana*) and a suspension of 3×10^6 conidia/mL was prepared as described in Chapter three. An aliquot of 0.1mL of the suspension was transferred on SDA (for *M. anisopliae*) or PDA (for *B. bassiana*) in 95 mm (diameter) × 15 mm (height) plastic Petri dishes and spread-plated using a sterile L-shaped looped glass rod. Plated Petri dishes were sealed with parafilm and incubated at 12, 16, 20, 24, 28, 32, and 36°C in the dark for 18 h. The experiment was replicated five times for temperature–isolate combination. Cultures were stained with 2mL lactophenol cotton blue and four pieces of 22 × 22 mm microscope coverslips were placed on stained culture. Conidial germination was examined microscopically at ×400 magnification by randomly counting 100 conidia (Figure 4.1). A conidium was considered germinated if it had a germination tube longer than its width. Conidial germination percentage was calculated using *Eqn* 3.1 in Chapter three.

4.2.2. Determination of the effect of temperature on mycelial growth

Metarhizium anisopliae and *B. bassiana* isolates were cultured as described in Section 4.2.1 with modification. Inoculated Petri dishes were maintained in the dark at 25 ± 2°C for 3 days. Two lines intersecting perpendicularly at the centre were drawn at the bottom of the Petri dish containing fresh media. Cylindrical media plug from the centre of the Petri dish was cut with a sterile 8-mm-diameter cork borer and replaced with cylindrical plugs cut from the three-day-old non-sporulated mycelial mat. Inoculated Petri dishes were sealed and incubated at 12, 16, 20, 24, 28, 32, and 36°C for 15 days in the dark. Five replicates of an isolate-temperature combination were prepared. Mycelial growth was determined by measuring the growth radially at 24 h intervals along two previously drawn cardinal lines (Figure 4.1). Radial growth of the isolates was plotted against time and fitted to the linear regression model ($y = \beta x + c$) and absolute growth rate (regression slope; mm/day) obtained using Microsoft Excel for the subsequent analyses.

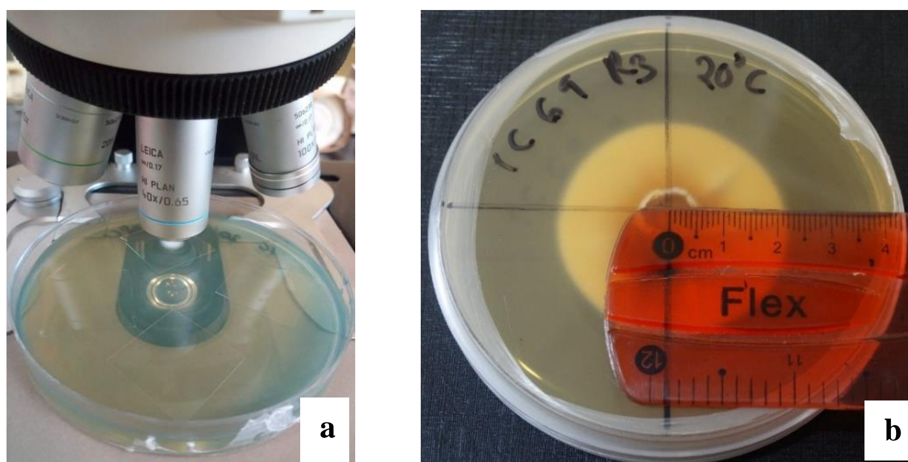


Figure 4.1: Representation of (**a**) conidial viability assessment by enumerating percentage germination of conidia and (**b**) measuring of daily radial growth of the fungus along two cardinal lines intersecting perpendicularly at the bottom centre of Petri dishes.

Note: Conidial viability and radial growth of six entomopathogenic isolates were measured at constant temperatures of 12, 16, 20, 24, 28, 32, and 36°C.

4.2.3. Nonlinear models for the study

The selection of nonlinear models for this study was based on the ability to describe and predict at least three biologically significant and physically interpretable parameters *viz.* minimum, optimum, and maximum thermal thresholds as well as maximal growth response of EPF at specific temperatures. Eight models from the literature were selected to describe the effect of temperatures on conidial germination and mycelial growth rate. These models have previously been used to quantitively describe microbial growth. These models are:

Brière 1 (Brière *et al.*, 1999): $\mu m = aT(T - Tmin)(Tmax - T)^{\frac{1}{2}}$ *Eqn* 4.1

$$a = \frac{Pmax}{Topt(Topt - Tmin)(Tmax - Topt)^{\frac{1}{2}}}$$ **Eqn* 4.2

Brière 2 (Brière *et al.*, 1999): $\mu m = aT(T - Tmin)(Tmax - T)^{\left(\frac{1}{b}\right)}$ *Eqn* 4.3

$$a = \frac{Pmax}{Topt(Topt - Tmin)(Tmax - Topt)^{\frac{1}{b}}}$$ **Eqn* 4.4

Ratkowsky 2 (Ratkowsky *et al.*, 1983):

$$\mu m = b(T - Tmin)\{1 - exp[c(T - Tmax)]\}^2$$

Eqn 4.5

$$b = \frac{Pmax}{(Topt - Tmin)\{1 - exp[c(T - Tmax)]\}^2}$$ **Eqn* 4.6

Ratkowsky 3 (Zwietering *et al.*, 1991):

$$\mu m = [b(T - Tmin)]^2\{1 - exp[c(T - Tmax)]\}$$

Eqn 4.7

$$b = \frac{Pmax}{(Topt - Tmin)^2\{1 - exp[c(T - Tmax)]\}} \qquad *Eqn\ 4.8$$

Lactin 1 (Lactin *et al.*, 1995): $\mu m = a(T - Tmin)^2(Tmax - T)$ *Eqn* 4.9

$$a = \frac{Pmax}{(Topt - Tmin)^2(Tmax - Topt)} \qquad *Eqn\ 4.10$$

Van Der Heide (Van Der Heide *et al.*, 2006):

$$\mu m = aT(T - Tmin)(Tmax - T) \qquad Eqn\ 4.11$$

$$a = \frac{Pmax}{Topt(Topt - Tmin)(Tmax - Topt)} \qquad *Eqn\ 4.12$$

CTMI (Rosso *et al.*, 1995):

$$\mu m = \frac{\mu opt(T - Tmin)(T - Tmin)^2}{Topt - Tmin[(Topt - Tmin)(T - Topt) - (Topt - Tmax)(Topt + Tmin - 2T)]} \qquad Eqn\ 4.13$$

Generalized β function (Bassanezi *et al.*, 1998):

$$\mu m = TYopt\left(\frac{T - Tmin}{Topt - Tmin}\right)^{c\frac{Topt - Tmin}{Tmax - Topt}}\left[\frac{Tmax - T}{Tmax - Topt}\right]^{c} \qquad Eqn\ 4.14$$

In the models' equations, the notation μm represents conidial germination (%) or mycelial growth (mm/day) at constant temperatures (T). T_{min}, T_{opt}, and T_{max} are the hypothetical lower minimum, optimum, and maximum temperatures, respectively, while P_{max} is maximal conidial germination or mycelial growth at T_{opt}. Parameter μ_{opt} in *Eqn* 4.13 is equivalent to P_{max}. Equations with asterisks (*) are reparameterizations of the original models to provide biologically significant parameters T_{opt} and P_{max} (Adams *et al.*, 2017). In *Eqn* 4.1 and *Eqn* 4.3, *a* is a redundant parameter while *b* in *Eqn* 4.3 is a curve-fitting parameter (Brière *et al.*, 1999). In *Eqn* 4.5 and *Eqn* 4.7, parameters *b* and *c* are the Ratkowsky parameters $°C^{-1}\ h^{-0.5}$ and $°C^{-1}$, respectively, where *b* represents the regression coefficient of the square root of the germination or

growth rate and c is a curve-fitting parameter (Ratkowsky *et al*., 1983). In *Eqn* 4.14, c is a shape parameter. Parameter a in *Eqn* 4.9 and *Eqn* 4.11 is a redundant parameter.

4.2.4. Data analysis

Modelling of conidial germination and mycelial growth rate as a function of temperature was performed using R software (R Core Team, 2020) by fitting the datasets to the models' formulae. The start values for each model–isolate's parameter required to achieve convergence tolerance were based on notional estimations. A nonlinear *nls* function was adopted for datasets with non-zero residual sum of squares and the *nlsLM* function from the *minpack.lm* package (Elzhov *et al*., 2016) was used for datasets with zero residual sum of squares. These mathematical functions provided weighted least-square estimates of the model's parameters for each isolate. The Akaike information criterion (AIC), adjusted R squared (adj. R^2) and physical interpretation of graphs plotted from models' predicted values were criteria used for comparisons and selection of the best fitting models. The accuracy of the models was assessed using the *rcompanion* package (Mangiafico, 2020) which provided a coefficient of determination (R^2). Adjusted R squared was calculated from R^2 using an equation developed by Kvålseth (1985):

$$R^2_{adj} = 1 - (1 - R^2)\left(\frac{n-1}{n-k-1}\right) \qquad \textit{Eqn } 4.15$$

In this equation, n and k are the numbers of samples and model parameters, respectively. Models with the lowest AIC, highest adj. R^2 values and curves accurately describing the data were considered as best fitting. Single best-fitting models for each isolate were based on AIC and adj. R^2 and equally best-fitting models were established according to Vuong's (1989) likelihood ratio test for comparing non-nested models. This likelihood ratio test is applied in the *nonnest2* package (Merkle and You, 2020).

4.3. Results

4.3.1. Comparison of the models for conidial germination

The fitted graphs of conidial germination against temperature showed obvious nonlinear patterns (Figure 4.2). The goodness-of-fit statistics for the models describing temperature-conidial germination relationships for the isolates are provided in Table 4.1. According to goodness-of-fit, the suitability of models varied across the EPF isolates for conidial germination. According to the likelihood ratio test, the least and best-fitting models for conidial germination are presented in Table 4.2. For conidial germination, CTMI was the best-fitting model while Ratkowsky 3 was the equally best-fitting model for all isolates. Lactin 1 was an equally best-fitting model for conidial germination of ICIPE 7 and ICIPE 20, while Ratkowsky 2 was an equally best-fitting model for conidial germination of ICIPE 20 and ICIPE 284. Briere 2 and Van Der Heide were also equally best-fitting models for conidial germination of ICIPE 20.

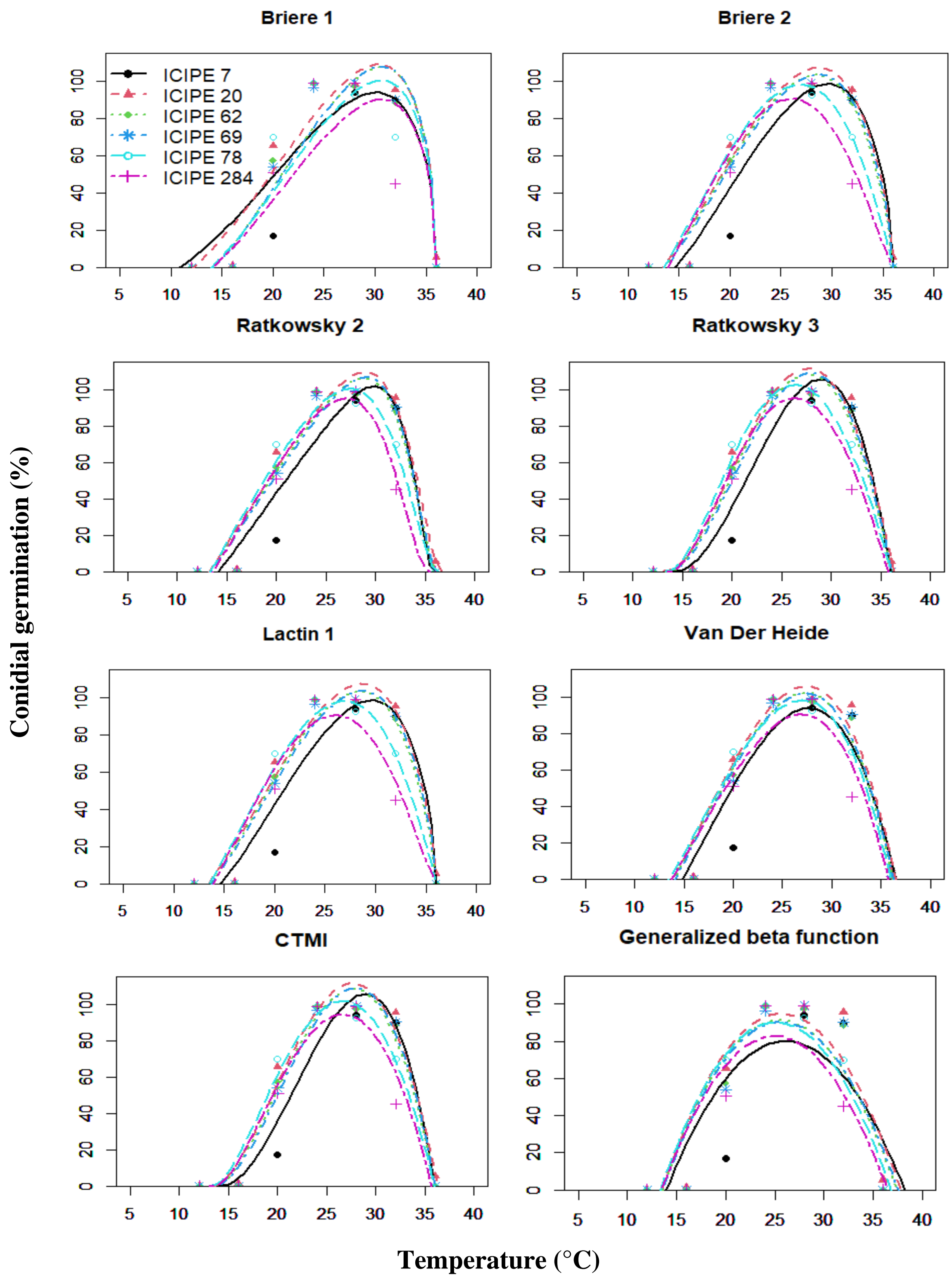


Figure 4.2: Curves of nonlinear models predicting the effect of temperature on conidial germination of *Metarhizium anisopliae* and *Beauveria bassiana*. CTMI = cardinal temperature model with inflection.

Table 4.1: Comparison of nonlinear models used to predict the effect of temperature on conidial germination of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284.

Model[a]	Criterion[b]	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	AIC	69.62	65.19	65.45	64.22	69.96	72.91
	Adj. R^2	0.57	0.76	0.75	0.79	0.48	0.17
Brière 2	AIC	70.45	62.45	65.01	63.33	65.81	74.32
	Adj. R^2	0.27	0.68	0.65	0.72	0.57	-0.52
Ratkowsky 2	AIC	70.91	66.05	65.69	64.97	66.71	67.05
	Adj. R^2	0.21	0.60	0.61	0.65	0.51	0.46
Ratkowsky 3	AIC	66.51	62.18	57.65	58.27	60.09	63.43
	Adj. R^2	0.59	0.77	0.84	0.87	0.81	0.68
Lactin 1	AIC	65.42	63.48	62.43	60.89	66.58	69.05
	Adj. R^2	0.76	0.82	0.84	0.84	0.68	0.52
Van Der Heide	AIC	71.22	64.83	64.55	64.84	63.79	66.77
	Adj. R^2	0.46	0.78	0.78	0.78	0.79	0.66
CTMI	AIC	64.51	60.08	57.63	56.33	59.02	62.91
	Adj. R^2	0.79	0.89	0.92	0.93	0.89	0.80
Generalized β function	AIC	78.51	74.43	74.65	75.09	72.21	73.50
	Adj. R^2	-0.16	-0.33	-0.40	-0.47	-0.07	-0.36

[a] Temperature-dependent model: CTMI = cardinal temperature model with inflection.
[b] Selection criterion: AIC = Akaike information criterion, Adj. R^2 = adjusted R–squared.

Table 4.2: Likelihood ratio test (z values) between the best-fitting model and other models for conidial germination of the *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *Beauveria bassiana* (ICIPE 284) isolates.

Model[a]	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	2.25 *	1.67 *	2.52 *	2.58 *	3.31 *	7.48 *
Brière 2	2.45 *	0.78 ns	1.84 *	1.69 *	3.65 *	2.78 *
Ratkowsky 2	3.36 *	1.35 ns	2.38 *	2.41 *	6.47 *	0.72 ns
Ratkowsky 3	-3.13 ns	0.75 ns	0.14 ns	0.32 ns	-2.24 ns	-1.81 ns
Lactin 1	0.64 ns	1.47 ns	2.17 *	2.00 *	4.62 *	7.91 *
Van Der Heide	2.11 *	1.59 ns	2.31 *	2.67 *	3.82 *	2.92 *
CTMI	–	–	–	–	–	–
Generalized β function	3.73 *	4.39 *	5.90 *	6.58 *	6.42 *	2.71 *

The values presented are z statistics. The z statistics (–) of the single best-fitting model have not been presented. “ns”: non-significant ($p > 0.05$) indicating the model is equally fit, “*”: significant ($p < 0.05$) indicating the model is not equally fit.
[a] Temperature-dependent model: CMTI = cardinal temperature model with inflection.

4.3.2. Comparison of the models for mycelial growth

Graphically, mycelial growth of the isolates incubated at different temperature regimes exhibited unimodal and nonlinear growth (Figure 4.3). According to goodness-of-fit, the best fitting models varied across the isolates (Table 4.2). The least- and best-fitting models for mycelial growth according to the likelihood ratio test are presented in Table 4.4. For mycelial growth, CTMI best fitted ICIPE 7, ICIPE 20, ICIPE 69 and ICIPE 78, while Lactin 1 and Ratkowsky 2 best fitted ICIPE 62 and ICIPE 284, respectively. However, some models were equally best-fitting for mycelial growth of ICIPE 7 (Lactin 1, Ratkowsky 3, Brière 1, Brière 2 and Van Der Heide), ICIPE 20 (Ratkowsky 3), ICIPE 62 (CTMI and Ratkowsky 3), ICIPE 69 (Lactin 1, Ratkowsky 2, Ratkowsky 3 and Van Der Heide) and ICIPE 78 (Lactin 1, Ratkowsky 2, Ratkowsky 3, Brière 2 and Van Der Heide). Except for the above-mentioned best-fitting and equally fit models for each isolate, the other models poorly described mycelial growth of the isolates as a function of temperature.

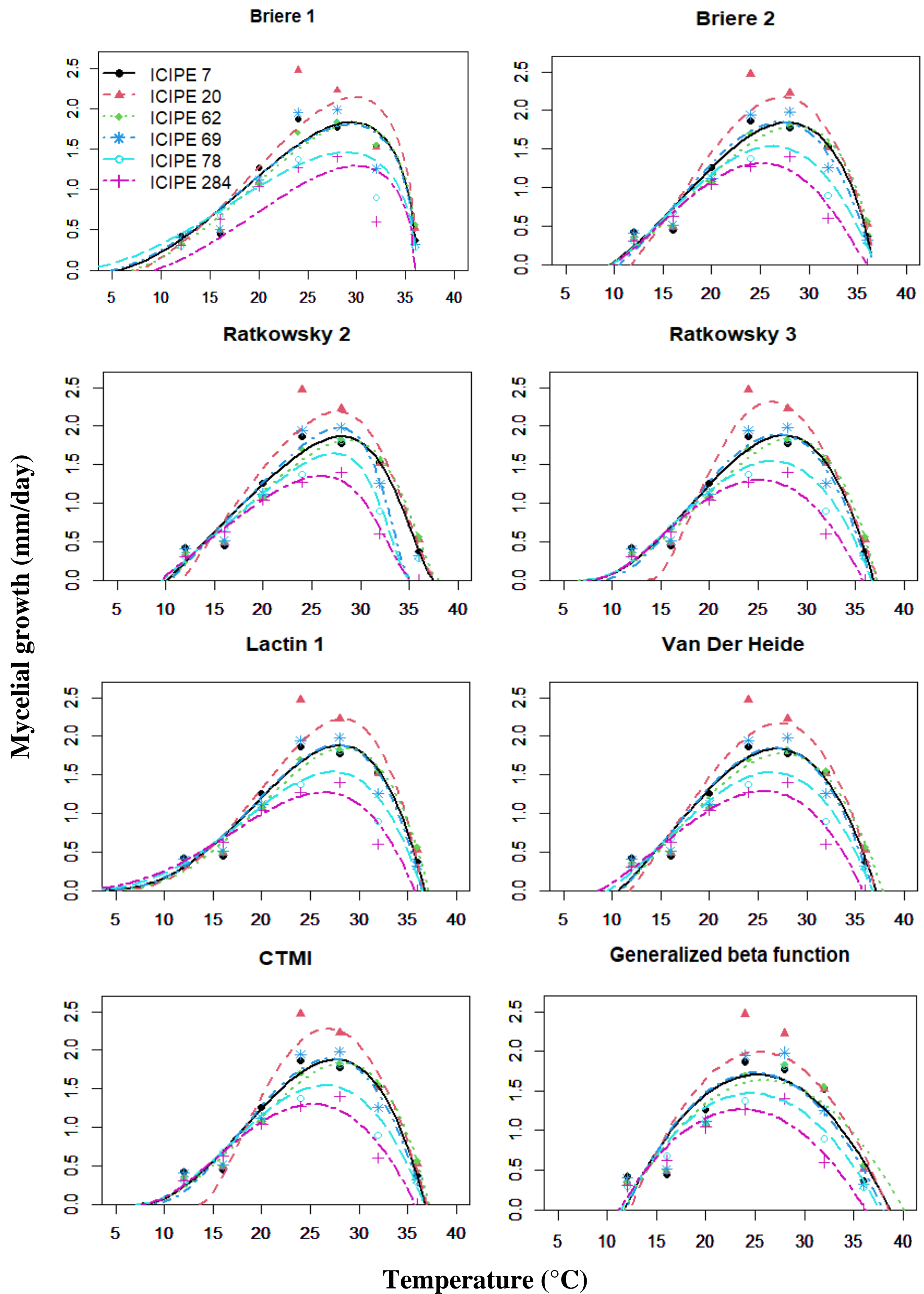


Figure 4.3: Curves of nonlinear models predicting the effect of temperature on mycelial growth of *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, and ICIPE 78) and *Beauveria bassiana* (ICIPE 284). CTMI = cardinal temperature model with inflection.

Table 4.3: Comparison of nonlinear models used to predict the effect of temperature on mycelial growth of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284.

Model[a]	Criterion[b]	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	AIC	6.37	15.62	0.85	10.93	9.35	8.70
	Adj. R^2	0.73	0.39	0.86	0.53	0.41	0.36
Brière 2	AIC	5.35	14.23	-0.03	8.35	5.74	8.04
	Adj. R^2	0.65	0.25	0.81	0.51	0.47	0.12
Ratkowsky 2	AIC	6.95	14.52	1.04	8.15	4.82	-6.95
	Adj. R^2	0.60	0.21	0.78	0.52	0.54	0.90
Ratkowsky 3	AIC	4.24	8.17	-2.67	6.77	5.54	0.27
	Adj. R^2	0.69	0.68	0.86	0.60	0.48	0.71
Lactin 1	AIC	2.32	12.27	-5.55	5.62	4.58	1.41
	Adj. R^2	0.82	0.60	0.93	0.78	0.70	0.77
Van Der Heide	AIC	4.58	12.24	1.38	6.47	7.20	-0.95
	Adj. R^2	0.79	0.63	0.84	0.75	0.64	0.84
CTMI	AIC	2.26	7.34	-4.50	4.78	3.54	-1.59
	Adj. R^2	0.85	0.81	0.93	0.80	0.74	0.85
Generalized β function	AIC	14.07	19.00	12.66	15.07	10.07	5.11
	Adj. R^2	-0.24	-0.48	-0.17	-0.29	0.02	0.42

[a] Temperature-dependent models: CTMI = cardinal temperature model with inflection.
[b] Selection criterion: AIC = Akaike information criterion, Adj. R^2 = adjusted R–squared.

Table 4.4: Comparison between the best-fitting model and other models for mycelial growth of the *Metarhizium anisopliae* (ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78) and *Beauveria bassiana* (ICIPE 284) isolates.

Model[a]	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	1.50 ns	3.10 *	2.52 *	2.12 *	3.91 *	5.43 *
Brière 2	1.39 ns	3.46 *	3.69 *	1.72 *	0.31 ns	3.60 *
Ratkowsky 2	1.89 *	1.85 *	4.81 *	-0.73 ns	-1.51 ns	–
Ratkowsky 3	-0.24 ns	-0.92 ns	0.80 ns	-0.06 ns	-0.16 ns	5.47 *
Lactin 1	0.04 ns	2.37 *	–	0.37 ns	0.72 ns	3.61 *
Van Der Heide	2.27 *	3.30 *	2.52 *	1.48 ns	1.04 ns	3.63 *
CTMI	–	–	0.78 ns	–	–	4.88 *
Generalized β function	5.82 *	3.92 *	6.02 *	3.76 *	1.81 *	6.18 *

The values presented are z statistics. The z statistics (–) of the single best-fitting model have not been presented. “ns”: non-significant ($p > 0.05$) indicating the model is equally fit, “*”: significant ($p < 0.05$) indicating the model is not equally fit.
[a] Temperature-dependent model: CMTI = cardinal temperature model with inflection.

4.3.3. Cardinal estimates for conidial germination and mycelial growth

Conidial germination of the isolates occurred between 10.9 to 38.3°C with the optima at 25.0 to 30.4°C and maximal conidial germination of 82.7 to 100.0% (Table 4.5). On the other hand, mycelial growth of the isolates occurred between –5.1 to 40.1°C with the optimal growth occurring between 23.7 to 29.8°C with maximal mycelial growth rates ranging between 1.17 to 2.31 mm/day (Table 4.6).

Predictive models provided different estimates for T_{min} and T_{max}. Unlike the other models, Brière 1 had the shortest range between T_{opt} and T_{max} for conidial germination; 30.2 to 36.0°C and mycelial growth; 29.0 to 36.2°C. This indicated a sharp decline in the rates of conidial germination and mycelial growth at temperatures above the T_{opt}. Brière 1 also predicted the same T_{max} estimates for conidial germination and mycelial growth across the EPF isolates. Comparatively, Brière 1 and the generalized β function gave a wide range of values for T_{min} (conidial germination; 10.8 to 12.7°C, mycelial growth; -5.1 to 7.8°C) and T_{max} (conidial germination; 36.5 to 38.3°C, mycelial growth; 36.2 to 40.1°C), respectively. Lactin 1 model provided a wider T_{min} range for mycelial growth (-2.9 to 4.6°C) than for conidial germination (11.2 to 12.8°C). The other models had relatively small ranges but variable estimates of T_{min} and T_{max}.

According to the best-fitting models, T_{opt} for conidial germination of ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and ICIPE 284 were 28.9, 27.6, 27.7, 27.9, 26.6 and 26.6°C, respectively with P_{max} of 100.0% except for ICIPE 284 (95.4%). The thermal requirements for germination of EPF isolates had low differences at T_{min} (14.2 to 13.2°C) and T_{max} (36.3 to 35.7°C). The faster growth rate (P_{max}) at T_{opt} was evident in ICIPE 20 (2.28 mm/day, 26.8°C) followed by ICIPE 69 (1.89 mm/day, 27.1°C),

ICIPE 7 (1.89 mm/day, 27.6°C), ICIPE 62 (1.85 mm/day, 28.4°C), ICIPE 78 (1.54 mm/day, 26.5°C) and lowest in ICIPE 284 (1.36 mm/day, 25.9°C).

Table 4.5: Models' estimates for conidial germination of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284.

Model	Parameter	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	P_{max}	98.9	100.0	100.0	100.0	96.5	86.8
	T_{min}	11.9	12.3	12.4	12.8	10.8	10.9
	T_{opt}	30.3	30.3	30.4	30.4	30.2	30.2
	T_{max}	36.0	36.0	36.0	36.0	36.0	36.0
Brière 2	P_{max}	99.0	100.0	100.0	100.0	98.4	100.0
	b	1.75	1.34	1.31	1.36	0.10	0.90
	T_{min}	13.5	13.5	13.2	13.8	13.5	12.0
	T_{opt}	29.8	28.6	28.4	28.7	27.0	26.0
	T_{max}	36.0	36.1	36.0	36.0	36.0	36.0
Ratkowsky 2	P_{max}	100.0	100.0	100.0	100.0	100.0	95.2
	c	0.45	0.30	0.32	0.34	0.23	0.24
	T_{min}	14.2	13.2	13.4	13.5	13.3	13.7
	T_{opt}	29.8	28.9	28.9	29.1	27.4	27.0
	T_{max}	35.9	36.7	36.3	36.2	36.3	35.4
Ratkowsky 3	P_{max}	100.0	100.0	100.0	100.0	100.0	95.4
	c	0.01	-0.04	-0.04	-0.03	-0.01	-0.09
	T_{min}	14.2	13.2	13.4	13.4	13.4	13.6
	T_{opt}	28.9	27.7	27.7	28.0	26.4	26.3
	T_{max}	36.0	36.3	36.1	36.1	36.0	35.7
Lactin 1	P_{max}	100.0	100.0	100.0	100.0	100.0	91.3
	T_{min}	12.8	9.2	11.6	10.0	7.5	7.7
	T_{opt}	29.4	28.9	28.7	28.9	28.2	28.1
	T_{max}	35.9	36.1	35.9	36.0	35.8	35.7
Van Der Heide	P_{max}	93.8	100.0	100.0	100.0	98.4	90.5
	T_{min}	14.9	13.9	14.0	14.1	13.5	13.8
	T_{opt}	27.7	27.3	27.3	27.4	27.1	27.0
	T_{max}	36.5	36.5	36.2	36.3	36.0	35.7
CTMI	μopt	100.0	100.0	100.0	100.0	100.0	94.4
	T_{min}	14.2	13.3	13.4	13.4	13.2	13.4
	T_{opt}	28.9	27.6	27.7	27.9	26.6	26.7
	T_{max}	36.0	36.3	36.1	36.0	36.0	35.7
Generalized β function	P_{max}	79.5	94.8	90.9	90.3	90.3	82.8
	c	-0.11	0.32	0.74	-0.86	0.11	1.32
	T_{min}	14.0	13.5	13.6	13.6	13.4	13.5
	T_{opt}	25.2	25.3	25.0	25.2	25.3	25.3
	T_{max}	38.3	37.8	37.5	37.6	36.8	36.5

[a] Temperature-dependent model: CMTI = cardinal temperature model with inflection.
[b] Model's parameters: b and c are empirical parameters; T_{min}, T_{opt} and T_{max} are minimum, optimum and maximum temperatures (°C); P_{max} and μ_{opt} are maximal germination (%) at T_{opt}. The original models' redundant parameter a in Brière 1, Brière 2 and Van Der Heide, and b in Ratkowsky 2 and Ratkowsky 3 are not presented, reparametrized to provide P_{max} and T_{opt}.

Table 4.6: Models' estimates for mycelial growth for *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 62, ICIPE 69, ICIPE 78, and *Beauveria bassiana* ICIPE 284.

Model	Parameter	IC 7	IC 20	IC 62	IC 69	IC 78	IC 284
Brière 1	P_{max}	1.83	2.14	1.81	1.80	1.46	1.17
	T_{min}	5.6	7.7	7.2	5.0	0.5	7.8
	T_{opt}	29.4	29.7	29.8	29.4	29.1	28.1
	T_{max}	36.1	36.1	36.3	36.1	36.1	36.0
Brière 2	P_{max}	1.84	2.17	1.80	1.85	1.54	1.50
	b	1.26	1.03	1.41	1.07	0.95	1.00
	T_{min}	9.6	11.7	9.6	10.5	9.8	10.0
	T_{opt}	27.9	27.3	28.6	27.0	26.4	26.0
	T_{max}	36.5	36.9	36.7	36.6	36.8	36.0
Ratkowsky 2	P_{max}	1.86	2.19	1.82	1.98	1.56	1.36
	c	0.23	0.19	0.24	0.34	0.18	0.22
	T_{min}	10.1	11.7	10.2	10.5	10.0	9.5
	T_{opt}	28.1	27.6	28.7	27.7	26.7	25.9
	T_{max}	37.7	37.9	38.2	35.2	37.5	35.4
Ratkowsky 3	P_{max}	1.88	2.31	1.84	1.89	1.55	1.30
	c	0.02	-0.13	0.05	-0.01	-0.01	-0.04
	T_{min}	7.3	13.8	6.7	8.7	7.3	7.6
	T_{opt}	27.7	26.4	28.5	27.0	26.5	25.1
	T_{max}	36.8	37.3	37.2	36.6	36.7	35.8
Lactin 1	P_{max}	1.89	2.22	1.85	1.85	1.54	1.27
	T_{min}	2.8	4.7	3.7	3.6	-0.3	-2.9
	T_{opt}	28.1	28.4	28.4	28.5	27.2	26.2
	T_{max}	36.7	36.6	37.2	37.1	36.5	35.7
Van Der Heide	P_{max}	1.84	2.16	1.78	1.85	1.53	1.29
	T_{min}	10.6	11.7	10.9	10.8	9.6	8.6
	T_{opt}	26.9	27.1	27.4	26.7	26.5	25.6
	T_{max}	37.1	37.0	37.8	36.8	36.7	35.7
CTMI	μ_{opt}	1.89	2.28	1.83	1.89	1.54	1.30
	T_{min}	7.4	13.5	7.0	8.7	7.3	7.5
	T_{opt}	27.6	26.8	28.4	27.1	26.5	25.2
	T_{max}	36.8	37.0	37.2	36.6	36.7	35.8
Generalized β function	P_{max}	1.71	2.00	1.63	1.73	1.47	1.27
	c	0.32	0.45	-0.05	-0.06	-0.04	-0.17
	T_{min}	11.7	12.3	11.8	11.8	11.4	11.2
	T_{opt}	24.8	25.6	25.8	24.7	24.4	23.7
	T_{max}	38.7	38.6	40.1	38.1	37.7	36.2

[a] Temperature-dependent model: CMTI = cardinal temperature model with inflection.

[b] Model's parameters: b and c are empirical parameters; T_{min}, T_{opt} and T_{max} are minimum, optimum and maximum temperatures (°C); P_{max} and μ_{opt} are maximal mycelial growth (mm/day) at $T_{opt.}$ The original models' redundant parameter a in Brière 1, Brière 2 and Van Der Heide, and b in Ratkowsky 2 and Ratkowsky 3 are not presented, reparametrized to provide P_{max} and T_{opt}.

4.4. Discussion

Graphically, the temperature had a nonlinear effect on conidial germination and mycelial growth. Variations in the best-fitting models for the isolates indicate that a single model is not sufficient to forecast the germination and growth of several isolates as a function of temperature. This is because the EPF isolates have different cardinal requirements as characterised by different estimates for T_{min}, T_{opt}, and T_{max}.

The goodness-of-fit of the models may vary based on the number of model's parameters, fungal species and isolates, and the number of observations in datasets fitted to the models. For example, models with fewer parameters tend to have high fitness and vice versa (Zwietering *et al*., 1991). Whenever adjusted R^2 is used as a basis of models' comparison, the numbers of observations in datasets and model's parameters based on the equation by Kvålseth (1985) may affect models' variances. For example, the degree of explained variances (adjusted R^2) decreases with the number of observations in datasets fitted to a model. Different fungal species and isolates have different thermal requirements (Fargues *et al*., 1997; Ouedraogo *et al.*, 1997). Although some models have suitably described many fungal isolates, some isolates are best described by certain models (Davidson *et al*., 2003; Smits *et al*., 2003). Therefore, the suitability of models may vary based on these observations.

In this study, the best models to describe the effect of temperature on conidial germination of the tested isolates were CTMI, while the best models for mycelial growth were CTMI (for ICIPE 7, ICIPE 20, ICIPE 69 and ICIPE 78), Lactin 1 (for ICIPE 62) and Ratkowsky 2 (for ICIPE 284). Except for mycelial growth of ICIPE 284, Ratkowsky 3 could be used as an alternative model to describe the effect of

temperature on conidial germination and mycelial growth of the isolates. Other models showed some fitness specificity to the isolates.

The generalized β function was previously been used to describe the effect of temperature on mycelial growth of EPF (García–Fernández *et al*, 200; Quesada–Moraga *et al*., 2006). According to the authors, the model suitably described the thermal biology of *M. anisopliae* and *B. bassiana* isolates based on the coefficient of determination (R^2) and standard errors of the parameters, however, the model was not compared to other temperature-dependent models. In the present study, this model was relatively least–fitting for both conidial germination and mycelial growth for the tested EPF isolates. According to Zwietering *et al*. (1991), Ratkowsky 3 (a modified form of Ratkowsky 2) was a comparatively suitable model to describe the effect of temperature on bacterial growth. Despite Ratkowsky 3 being an alternative suitable model for conidial germination and mycelial growth of all tested isolates, Ratkowsky 2 was an alternative suitable model to describe the effect of temperature on some isolates (conidial germination; ICIPE 20, ICIPE 284, and mycelial growth; ICIPE 69 and ICIPE 78).

Smits *et al*. (2003) showed Brière 2 and Ratkowsky 3 best described the growth of *M. anisopliae* isolates and two isolates of *M. flavoviride* while Brière 1 was comparatively least–fitting model. In this study, Ratkowsky 3 was among the best-fitting models for both conidial germination and mycelial growth while Brière 1 and Brière 2 least fitted many conidial germination and mycelial growth datasets. Maybe the reparameterization of the models except for CTMI and the generalized β function to provide parameters T_{opt} and P_{max} may have contributed to less degree of fitness (Zwietering *et al*., 1991).

The effect of temperature on conidial germination has never been described using nonlinear models used herein. Best models show that *M. anisopliae* isolates have variable cardinal requirements at T_{min} (13.2–14.2°C), T_{opt} (26.6–28.9°C), T_{max} (36.0–36.3°C), and P_{max} of 100%, while T_{min}, T_{opt}, $T_{max,}$ and P_{max} of *B. bassiana* isolate was 13.4°C, 26.7°C, 35.7°C, and 94.4%, respectively. Thermotolerance of the EPF isolates according conidial germination were as follows: ICIPE 7 > ICIPE 69 > ICIPE 62 > ICIPE 20 > ICIPE 78 > ICIPE 284. For mycelial growth of *M. anisopliae* isolates, the cardinal requirements remained variable at T_{min} (3.6–11.6°C), T_{opt} (26.4–28.5°C), T_{max} (36.6–37.9°C) and P_{max} (1.54–2.28 mm/day) while *B. bassiana* isolate had the lowest cardinal requirements at T_{min} (9.4°C), T_{opt} (25.9°C), T_{max} (35.4°C), and P_{max} (1.36 mm/day). Thermotolerance of the fungal isolates according mycelial growth were as follows: ICIPE 62 > ICIPE 7 > ICIPE 20 > ICIPE 69 > ICIPE 78 > ICIPE 284. Temperature ranges required for conidial germination were shorter than those for mycelial growth while for $P_{max,}$ the optimal temperature required for conidial germination was slightly higher than those required for mycelial growth. This can be ascribed to high requirements of activation energy for enzymes involved in the germination process. Additionally, conidial germination of *M. anisopliae* and *B. Bassiana* isolates were comparatively less tolerant to low temperatures than mycelial growth, and this observation is consistent with previous studies (Bayissa *et al*., 2017; Tefera and Pringle, 2003 Ekesi *et al*., 1999). Although the tested EPF are mesophilic, specific thermal requirements for germination and growth varied among their species and isolates, and this can be ascribed to regions of origin.

The growth responses of EPF are best described at optimum temperatures where they have optimal performance (Davidson *et al*., 2003). Results from the single best-fitting models indicate that all the tested isolates have optimal germination around 26.6–

28.9°C and optimal growth around 25.9–28.4°C. This temperature range matches the geo-climatic conditions of most tropical countries. However, considering that these isolates can intentionally or unintentionally be introduced into bee colonies, their ability to operate maximally is unlikely to ensue owing to highly thermoregulated hive conditions (Jarimi *et al*., 2020). Although EPF showed a precipitous decline in growth at a temperature above T_{opt} with growth approaching zero near the T_{max}, further research is required to determine the effects of these EPF isolates under *in vivo* conditions of the honey bee or stingless bee colonies.

CHAPTER FIVE

5.0. EFFECT OF ENTOMOPATHOGENIC FUNGUS (*Metarhizium anisopliae*) ON SURVIVAL, POLLINATION BEHAVIOUR AND POLLINATION SUCCESS OF AFRICAN STINGLESS BEE (*Meliponula ferruginea*) POLLINATING CUCUMBER (*Cucumis sativus*)

Abstract

The effect of entomopathogenic fungi (EPF) on pollinators under field conditions is critical in understanding their environmental safety. Therefore, the effect of a novel EPF on survival, foraging behaviour, and success was evaluated on *Meliponula. ferruginea* pollinating cucumber *Cucumis sativus* under semi-field conditions. This study was conducted at the International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya. Cucumber plants were raised in two greenhouses divided into 4 treatment plots. Colonies of *M. ferruginea* were introduced into plots at the inception of flowering. Cucumber plants in two plots randomly designated as control plots were sprayed with sterile 0.05% Triton-X-100 while cucumber plants in biopesticide plots were sprayed with suspension (1×10^8 conidia/mL) of *M. anisopliae* ICIPE 69. Flight and foraging activity of bees, fruit set, and yield were recorded within 9 days before until 18 days after treatment application. Survival of forager bees was recorded every six days for 18 days after treatment application. Pollen load and conidial acquisition by bees and conidial persistence on plant surfaces were observed every three days for 18 days after treatment application. Experiments were repeated three times at different cucumber growing seasons. The biopesticide treatment did not significantly affect the flight activity, flower visitation, pollen foraging, fruit set, and yield neither did it affect the survival of the forager bees. The forager bees acquired significantly high levels of

conidia (7,600 ± 54 CFU/bee) immediately after biopesticide application followed by significant declines in the subsequent days. Conidial acquisition did not correlate with the pollen load on forager bees (657 ± 29 pollen/bee) during flowering periods.

5.1. Introduction

Stingless bees in Afrotropical regions are populous colonies in diverse ecosystems, pollinating various wild plants and cultivated crops, and producing high-quality honey (Bafo, 2019; Eardley and Kwapong, 2013; Slaa, *et al*., 2006; Eardley, 2004). They visit flowers of about 90 crops while effectively pollinating 18 crops (Slaa *et al*., 2006; Heard, 1999). They are preferred as pollinators because of their perennial colonies with high polylecty, ecological adaptability, floral constancy, effective forager recruitment and ease of domestication (Heard, 1999). Recently, species of stingless bee *Meliponula* are been used as pollinators to improve crop productivity in Africa, especially in Kenya (Kiatoko, *et al*., 2014; Asiko, 2012) and Uganda (Kajobe, 2006). However, stingless bee ecosystem services are at peril owing to the toxic effect of several chemical insecticides (Arena and Sgolastra, 2014). Therefore, the search for sustainable intervention for pollinator management is critically important.

Recently, the application of biopesticides is increasingly preferred because their merits outweigh the application of chemical insecticides which are toxic to their environment and which have resulted in the development of resistant pest populations (Kidanu and Hagos, 2020; Maina *et al*., 2018; Thungrabeab and Tongma, 2007). Although biopesticides are considered safe to nontarget organisms, field validation especially on the insect pollinators may be required before implementation to programmes such as integrated pest management (IPM) and integrated pest and pollinator management IPPM. In the fields, biopesticides applied on flowering crops may affect the

pollinators. The impact of fungal-based biopesticides especially on foraging behaviour, success, and survival of stingless bees in the field is yet to be investigated.

Cucumber *C. sativus* constitutes one of the crops in the family Cucurbitaceae and is a global leading crop with a high insect pollination requirement (Giannini *et al*., 2015; Klein *et al*., 2007). Yet fruit flies, whiteflies, aphids, and spider mites have been reported as devastating pests of crops in the family Cucurbitaceae (Kambura *et al*. 2018; Sharma *et al*. 2016). *Metarhizium anisopliae* ICIPE 69 represents 60% field coverage (80,000 ha) of fungal-based biopesticides developed at *icipe* for controlling several arthropod pests including fruit flies (Akutse *et al*., 2020). The present study aimed at assessing the effect of *M. anisopliae* ICIPE 69 sprayed on cucumber plants on the African stingless bee *M. ferruginea* survival, flight activity, foraging behaviour, fruit set and yield. Persistence of fungus on flowers and leaves under greenhouse conditions was also evaluated. This information is critical in IPPM programmes.

5.2. Material and Methods

5.2.1. Preparation of *Metarhizium anisopliae* ICIPE 69

A suspension of *M. anisopliae* ICIPE 69 (3×10^6 conidia/mL) was prepared as described in Chapter three (Section 3.2.2.) and a 0.1mL aliquot cultured in 50mL sterile Sabouraud dextrose broth (Oxoid) contained in a 250mL conical flask. The flask and its contents were incubated in an electric rotary shaker (Innova4, New Brunswick Scientific Co., Inc., New Jersey, US) adjusted to 25°C and 250 rpm. After 3 days of incubation in dark, the conidia developed into blastospores that were used as a starter culture for mass production.

Whole rice grains (2kg) were thoroughly washed with tap water and precooked at 80°C for 1 h. Precooked grains were placed in an autoclavable polythene bag and autoclaved at 121°C and 15 PSI for 35 min. Thereafter, the grains were cooled to room temperature and inoculated with blastospores. The bag with inoculated grains was sealed and incubated for 21 days at 25 ± 2°C and in the dark while being kneaded gently every two days to promote aeration and uniform colonization. Colonized grains were transferred to a 30 cm (diameter) × 15 cm (height) plastic tray and allowed to dry in a cold room (4°C) for 4 days in the dark. Conidia were harvested by agitating the colonized grains on 150–µm aperture mesh for 30 min. The powdery conidia were collected using a plastic tray and placed in sealable polythene bags. Bags were stored at 4°C in the darkroom before semi-field application.

5.2.2. *Meliponula ferruginea* colony

Stingless bee (*M. ferruginea*) colonies nested in Original Australian Trigona Hives (OATH) (40 × 25 × 22 cm) were collected from the International Centre of Insect Physiology and Ecology (*icipe*) meliponary established at Isiekuti village around the Kakamega Forest, Kenya (0°13'46" N 34°51'22" E). Each colony (approx. 3,000 bees) was visually checked for the absence of pests and pathogens.

5.2.3. Experimental setup

Experiments were conducted and repeated three times between April 2020–July 2020, August 2020–November 2020, and November 2020–January 2021. Two greenhouses (120 m^2) at *icipe*, Nairobi, Kenya (01°13′44″S, 36°54′16″E, 1,600 m above sea level) were used for the study. During the experiments, the average monthly temperature and relative humidity inside the greenhouses during midday (01:30 pm to 02: 30 pm) were 28.7 ± 0.6 and 40.4 ± 2.5%, 31.6 ± 0.6 and 40.0 ± 1.5%, 30.4 ± 0.4 and 41.6 ± 1.4%,

in the first, second and third, respectively. Five plots (3 × 8 m each) in each greenhouse were separated with insect-proof netting material (0.26–mm mesh size) (Figure 5.1).

Cucumber (*C. sativus*) plants were raised from seeds ('Ashley' variety, Simlaw Seeds Co., Nairobi, Kenya) in a nursery tray and the seedlings (14–day–old) were individually transplanted in red soil (nitisols) and well-decomposed goat manure mixed in 50% (v/v) proportion and contained in 23L plastic planting polybags. Thirty polybags were placed in each treatment plot, arranged in 10 rows and 3 columns, and set 0.8 m within rows and 0.9 m between rows according to good agricultural practices (GAP) (Figure 5.1). Plants were watered via drip irrigation. For each plant, three vines were trained to climb vertically installed 2.5 m trellis and extra branches were periodically trimmed. To promote flowering, each plant was top-dressed with 20g of 20:10:10 NPK fertilizer (MEA, Nairobi, Kenya) one week prior to blooming.

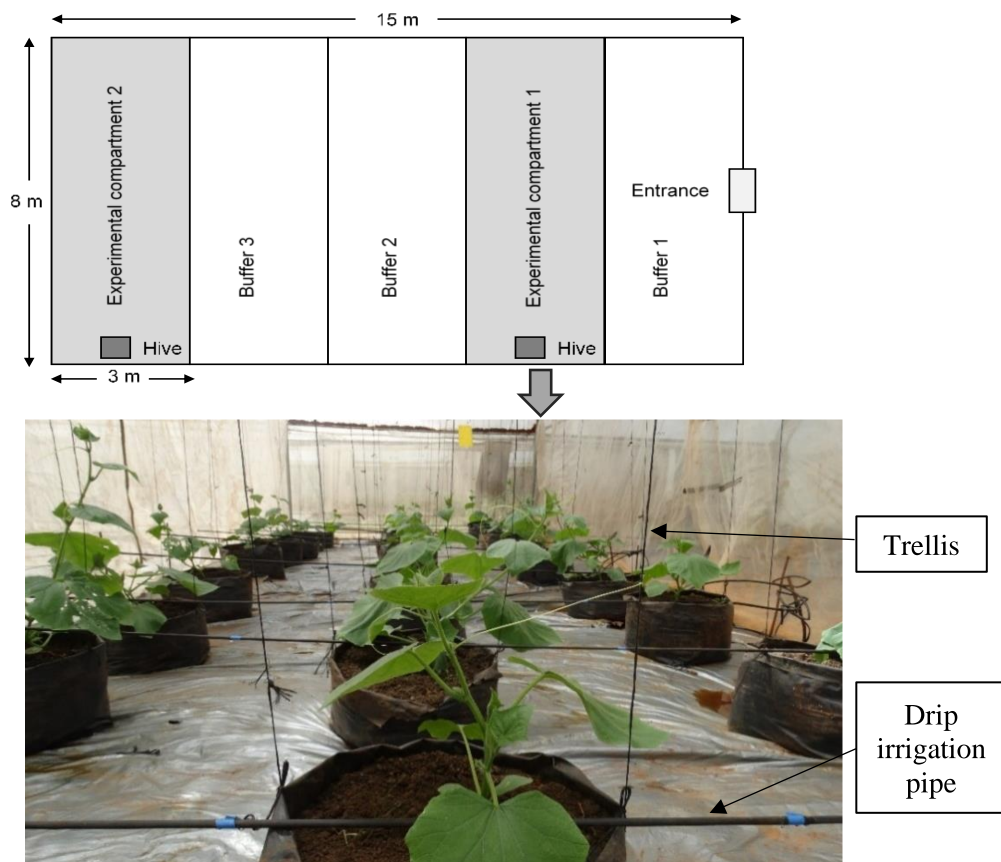


Figure 5.1: Experimental layout in greenhouses and one greenhouse compartment containing young cucumber *Cucumis sativus* plants.

Hive colonies of *M. ferruginea* (one hive per plot) were introduced to one side and in a similar position across treatment plots at the start of active flowering (Figure 5.2). Colonies of *M. ferruginea* were allowed to acclimatize to greenhouse conditions for 7 days before commencement of data collection. Prior to foraging, bees were artificially provided with distilled water to drink and propolis to build bee colony structures. Distilled water and propolis were loaded in two plastic plates that were placed 6 m from each colony. Distilled water and propolis were replenished daily and after five days, respectively. Two blue and two yellow sticky traps were installed in each treatment plot to trap any arthropod pests that may have gained access into the greenhouses. The sticky traps did not catch any bees.

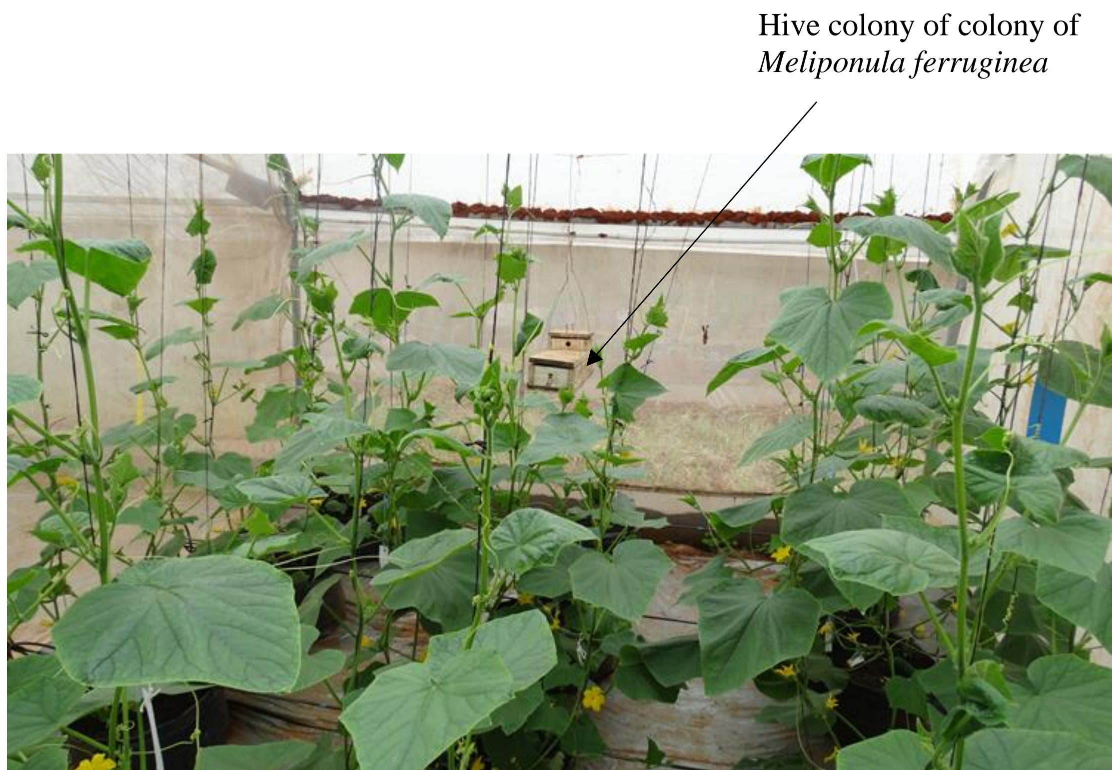


Figure 5.2: Representation of treatment plot containing blooming cucumber (*Cucumis sativus*) plants and installed with colony of *Meliponula ferruginea*.

5.2.4. The application of *Metarhizium anisopliae* ICIPE 69

Prior to application, conidial germination of *M. anisopliae* ICIPE 69 was assessed using the procedure described in Chapter three. Conidial germination was 87 ± 4% and the fungus was formulated into a field application rate of 1×10^8 conidia/mL in sterile 0.05% Triton-X-100. Cucumber plants were sprayed with 1.5L of the fungal suspension (biopesticide plots) or 0.05% Triton-X-100 (control plots) using a 16L knapsack hand spray pump (Copia, Nairobi, Kenya) standardized to deliver 300L/ha. Cucumber plants in the control treatment were sprayed first followed by those in the biopesticide treatment. Treatments were applied in the late evening (6:30 to 7:00 pm) when the bees were no longer flying in and out of the hives. Before treatment application, the bee colonies and feeders were temporarily removed from the plots. Treatments were applied 16 days after the installation of hive colonies.

5.2.5. Determination of flight intensity, foraging activity, and survival of bees

Flight activity was assessed by recording the instant number of bees entering and exiting the hive at 5 min intervals for 30 min between 1:30 pm to 2:00 pm. This observation was made simultaneously across all experimental plots and every three days starting 6 days before until 18 days after treatment application (i.e., –6, –3, 0, 3, 6, 9, 12, 15, and 18 days). Foraging activity was assessed by recording the number of bees visiting flowers of three focal plants at 5 min intervals for 30 min between 2:00 pm to 2:30 pm (Figure 5.3). The three plants were those occurring in the middle row across all treatment plots. Foraging activity was recorded simultaneously across all treatment plots and, thereafter, the number of opened flowers in these focal crops was recorded. These observations were made on 9 sampling dates indicated above. Observation on flight and foraging activity were made by trained personnel who were

randomly assigned to each treatment plot during each sampling dates to avoid observer biases.

Figure 5.3: Stingless bee (*Meliponula ferruginea*) foraging on a male flower (**a**) and a female flower (**b**) of cucumber (*Cucumis sativus*).

From each plot, 20 forager bees were collected for survival assay. Bees were gently captured on male flowers after foraging on pollen using clean plastic 50mL Falcon tubes and placed in 0.5L plastic cages. Bees were sampled at peak foraging time (2:30 pm to 3:00 pm) on 0, 6, 12, and 18 days after treatment application. Caged bees were maintained as described in Chapter three and their survival was recorded for 20 days.

5.2.6. Determination of fruit set, development, and yield

Female flowers in treatment plots were tagged at the date of inception 9 days before until 9 days after treatment application. Fruit set was determined 6 days from tagging and a fruit with > 6 cm length were considered successfully set and expressed as follows:

$$Fruit\ set\ (\%) = \frac{Number\ of\ formed\ fruits}{Total\ number\ of\ tagged\ female\ flowers} \times 100 \qquad Eqn\ 5.1$$

The number of fruits from tagged flowers reaching physiological maturity (14-day-old fruit, Figure 5.4) was recorded and expressed in percentage as follows:

$$Fruit\ maturation\ (\%) = \frac{Number\ of\ mature\ fruits}{Total\ number\ of\ tagged\ flowers} \times 100 \qquad Eqn\ 5.2$$

All 14-day-old fruits produced by the main vines of cucumber plants across all treatment plots were harvested and individually weighed in an electronic balance (UW6200H, Shimadzu Corporation, Kyoto, Japan) with 0.01g accuracy (Figure 5.4).

Figure 5.4: Physiologically mature cucumber *Cucumis sativus* fruit (14-day-old fruit, tagged with red thread) ready for harvest (**a**) and measurement in the laboratory (**b**).

5.2.7. Determination of conidial acquisition and pollen load by bees

Five forager bees were gently collected after foraging on pollen from each plot using clean Falcon tubes. Bees were collected on 0, 3, 6, 9, 12, 15, and 18 days after the application of biopesticide. Pollen and conidia load of individual bees were dislodged by placing the bee in 1mL of distilled water containing 0.05% Triton-X-100 then vortexed for 3 mins at 700 rpm. The number of pollen and conidia were enumerated using a haemocytometer placed under a light microscope adjusted to ×100 and ×400 magnifications, respectively.

5.2.8. Evaluation of persistence and viability of conidia on cucumber crop

From each plot, five male flowers and five leaves were randomly selected, cut, and individually placed in sterile 50mL Falcon tubes containing 5mL of sterile 0.05% Triton-X-100. Samples were vortexed and dislodged conidia enumerated using a haemocytometer. The suspensions from leaf samples were pooled and tested for conidial germination as described in Chapter three. The samples were collected on 0, 3, 6, 9, 12, 15, and 18 days after treatment application.

5.2.9. Data analysis

Data were analysed in R statistical software (R Core Team, 2020). Foraging activity and fruit yield datasets were subjected to a generalized linear model (GLM). Datasets of flight activity and conidial persistence on leaves had overdispersions with no zero counts and thus were subjected to Quasi–Poisson regression. Datasets of conidial retention on flowers and conidial acquisition by forager bees had overdispersions with the abundance of zero counts and thus were analysed with zero-inflated negative binomial regression. Experimental trial acted as a random effect variable in these analyses. Survival, fruit set, fruit maturation, and conidial viability datasets were subjected to logistic regression. Post-hoc analyses were performed using the *lsmeans* package (Lenth, 2015) with the Tukey method for adjustment of p-value probability. Pearson's correlation analysis was used to establish the relationship between conidia load and pollen load. Bees in one hive colony in the control treatment in the first experiment failed to forage, hence, were excluded from analyses.

5.3. Results

5.3.1. Flight activity, foraging activity, and survival of forager bees

Significant variation in flight activity was evident among experiments ($p < 0.0001$). Results of flight activity are shown in Figure 5.5. In the first experiment, flight activity was not statistically different between treatments ($p = 0.79$), however, statistical difference was evident among observation dates ($p < 0.0001$) and interactions of treatment and observation days ($p < 0.0001$). Significant difference ($p < 0.05$) in flight activity was notable on day –3, 12 and 18 with the biopesticide treatment having lower flight activity than the control treatment on day –3 and 12 but higher than the control treatment on day 18.

In the second experiment, flight activity differed significantly between treatments ($p = 0.0009$), among observation days ($p < 0.0001$) but not interactions ($p = 0.36$). Significant difference ($p < 0.05$) in flight activity was evident only on day –3 with the biopesticide treatment having higher flight activity than control treatment.

In the third experiment, flight activity did not differ significantly between treatments ($p = 0.19$), but significant difference was observed among observation days ($p < 0.0001$) and interactions of treatments and observation days ($p = 0.021$). With exception of day 3, there was no significant difference between treatment among observation days ($p > 0.05$). Specifically, the flight activity in the biopesticide treatment was lower than the control treatment on day 3.

In the control treatment, flight activity averaged 48 ± 3, 34 ± 4, and 26 ± 4 bees/5 min in the first, second, and third experiments, respectively. In the biopesticide treatment,

flight activity averaged 49 ± 10, 41 ± 5 and 23 ± 3 bees/5 min in the first, second, and third experiments, respectively.

Foraging activity varied among experiments ($p < 0.0001$). Results of foraging activity are represented in Figure 5.6. In the first experiment, foraging activity was significantly different between treatments ($p = 0.0024$), among observation dates ($p < 0.0001$) and their interactions ($p < 0.0001$). Significant difference ($p < 0.05$) in foraging activity was notable on day –6, –3, 0, 3 and 18 after treatment application. Specifically, foraging in the biopesticide treatment was lower compared to the control treatment on day –6, –3, 0 and 3, but higher compared to the control treatment on day 18.

In the second experiment, foraging activity differed significantly between treatments ($p < 0.0001$), among observation days ($p < 0.0001$) and but not between their interactions ($p = 0.095$). Foraging activity in the biopesticide treatment was statistically higher ($p < 0.05$) compared to the control treatment on day –6, –3, 0, 9, 15 and 18 after treatment application.

In the third experiment, foraging activity differed significantly between treatments ($p < 0.0001$), among observation days ($p < 0.0001$) and their interactions ($p = 0.0008$). Foraging activity in the biopesticide treatment was statistically lower ($p < 0.05$) compared to the control treatment on day –6, –3, 0 and 6 after treatment application.

In the first, second, and third experiments, foraging activity in the control treatment was 0.6 ± 0.1, 0.7 ± 0.1, and 0.7 ± 0.1 bees/flower/5 min, respectively. In the first, second, and third experiments, flight activity in the biopesticide treatment was 0.5 ± 0.1, 1.2 ± 0.2, and 0.5 ± 0.1 bees/flower/5 min, respectively.

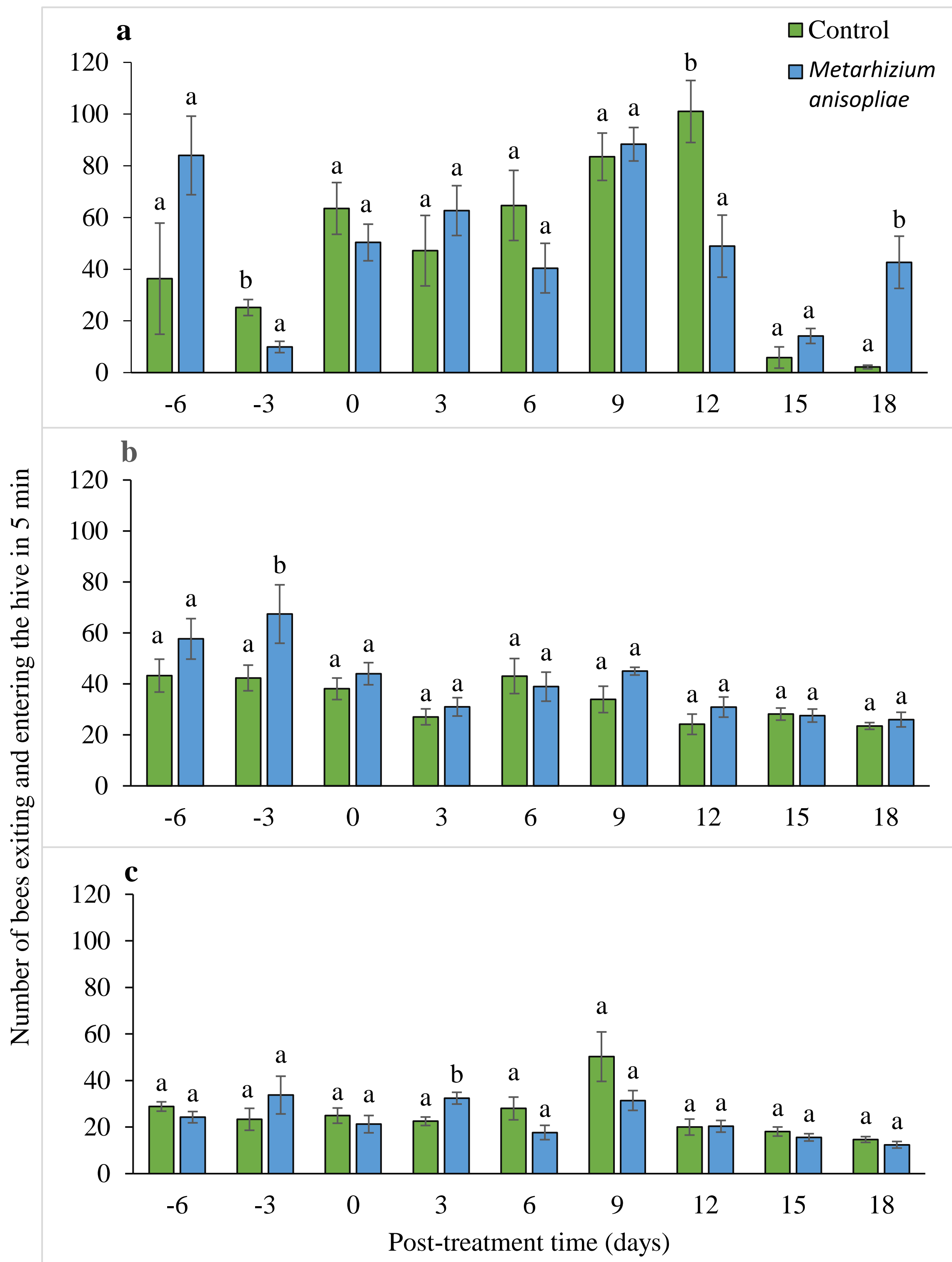


Figure 5.5: Flight activity of *Meliponula ferruginea* in treatment plots containing flowering *Cucumis sativus*.

Note: Error bars represent standard errors of the means. Same small letters above error bars indicate no significant differences between treatments according to the Turkey test at p=0.05. **a**: April 2020 – June 2020, **b**: September 2020 – November 2020, **c**: December 2020 – February 2021.

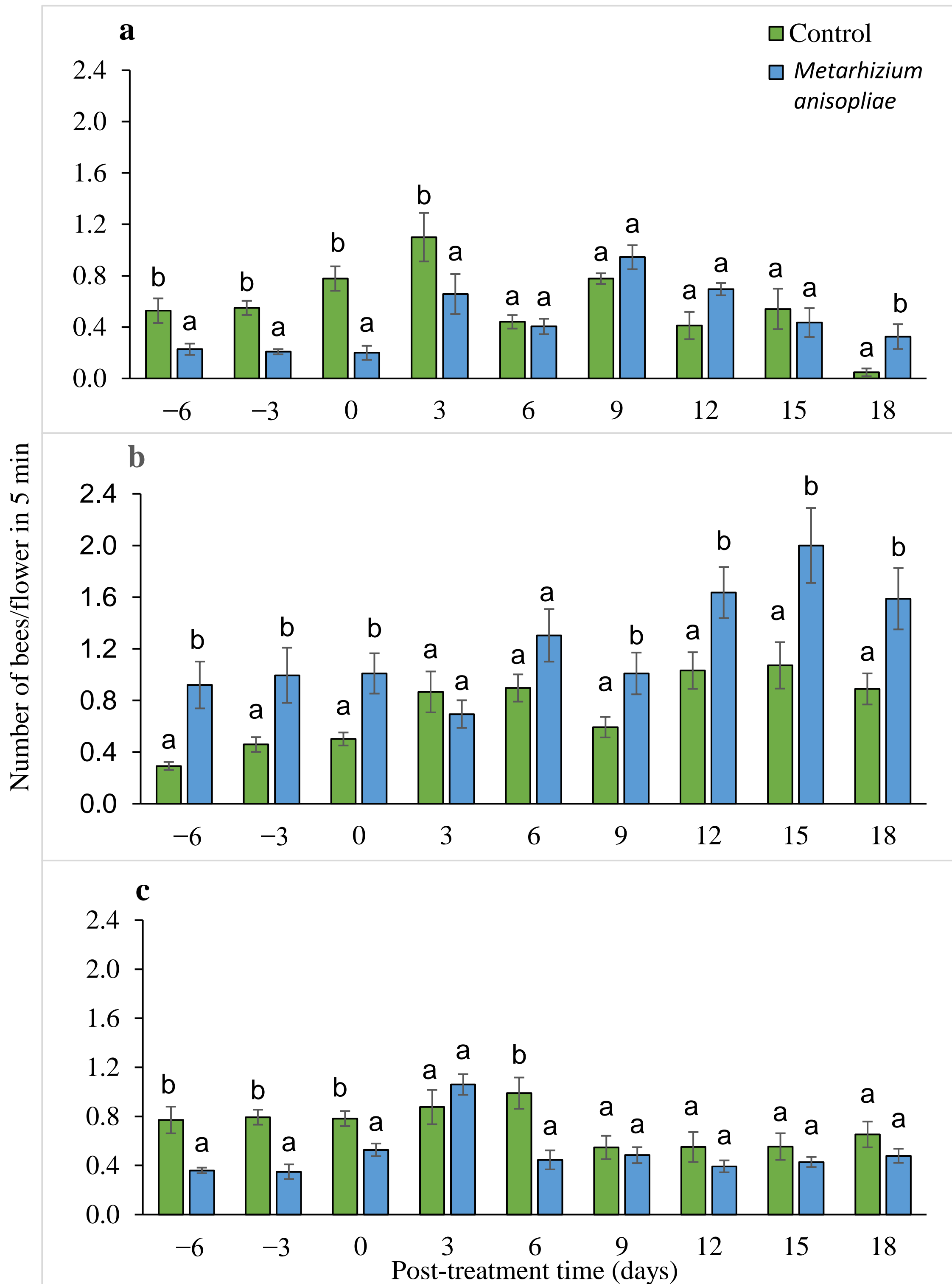


Figure 5.6: Foraging activity of *Meliponula ferruginea* in treatment plots containing flowering *Cucumis sativus*.

Note: Error bars represent standard errors of the means. Same small letters above error bars indicate no significant differences between treatments according to the Turkey test at p=0.05. **a**: April 2020 – June 2020, **b**: September 2020 – November 2020, **c**: December 2020 – February 2021.

Survival results of bee foragers are presented in Figure 5.7. Survival of forager bees were not statistically different among experiments (p = 0.92) neither was it affected by treatments (p = 0.11) or post-treatment days (p = 0.069). Numerically, survival of forager bees ranged between 84.2 to 96.0%. However, none of the forager bee cadavers had fungal development.

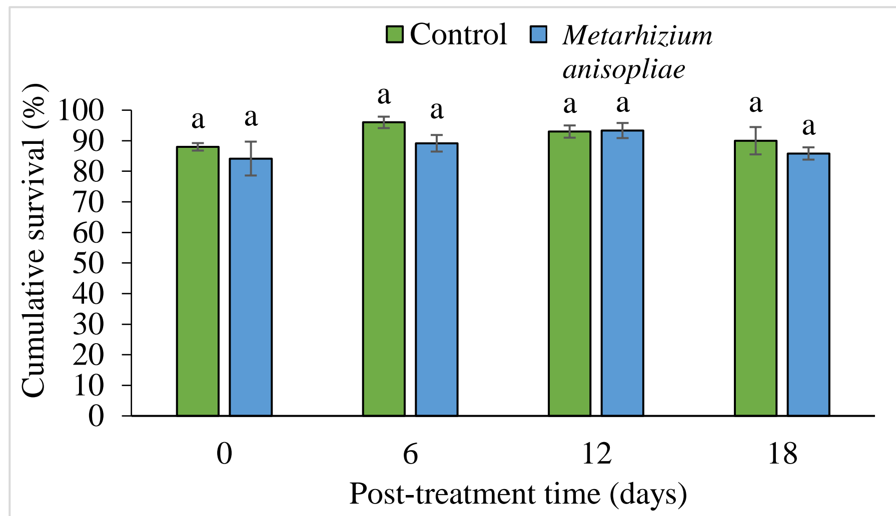


Figure 5.7: *Meliponula ferruginea* survival from plots containing treated *Cucumis sativus*.

Note: Error bars represent standard errors of the means. Different letters above error bars indicate significant differences according to the Turkey test.

5.3.2. Fruit set, development, and yield

Percentage of fruit set and mature fruit that resulted from the tagged cucumber flowers are presented in Table 5.1. Fruit set differed significantly across experiments (p = 0.20). However, there were no significant differences between treatments (p = 0.42), among observation days (p = 0.11) or their interactions (p = 0.76). Similarly, fruit maturation did not significantly differ among experiments (p = 0.64). Except between treatments (p = 0.55), the percentage of mature fruit was significantly different across observation days (p = 0.0099) and not in treatments*observation days interactions (p = 0.77). The mean fruit set was 91.1 ± 1.0% while fruit maturation was 84.8 ± 1.3%.

Table 5.1: Fruit set and maturation of *Cucumis sativus* pollinated by *Meliponula ferruginea* 9 days before until 9 days after treatment application.

Days	Fruit set (%)		Mature fruits (%)	
	Control	**Biopesticide**	**Control**	**Biopesticide**
−9 − −7	88 ± 2	89 ± 3	80 ± 4	80 ± 4
−6 − −4	94 ± 1	91 ± 2	88 ± 2	87 ± 2
−3 − −1	94 ± 1	89 ± 5	90 ± 2	87 ± 2
0 − 2	94 ± 2	96 ± 3	84 ± 5	88 ± 5
3 − 5	94 ± 1	92 ± 2	89 ± 2	88 ± 2
6 − 8	88 ± 1	86 ± 6	81 ± 2	76 ± 2
P-value	0.42		0.64	

The values (±) accompanying the means are standard errors. Biopesticide is *Metarhizium anisopliae* ICIPE 69.

Results of fruit weight are presented in Table 5.2. Fruit weight differed significantly across experiments ($p < 0.0001$). In the first experiment, fruit weight did not differ significantly between treatments ($p = 0.061$), however, the difference was noticed among the observation days ($p = 0.042$) but not between their interactions ($p = 0.78$). In the second experiment, fruit weight did not differ significantly between treatments ($p = 0.27$), among the observation days ($p = 0.68$) and their interactions ($p = 0.85$). In the third experiment, fruit weight was not significantly affected by treatments ($p = 0.52$), observation days ($p = 0.74$) and their interactions ($p = 0.2$). The mean values of fruit weight in the first, second and third experiments were 255.9 ± 23.0g, 357.1 ± 26.5g and 369.5 ± 22.3g, respectively.

Table 5.2: Fruit weight (g) of *Cucumis sativus* pollinated by *Meliponula ferruginea* at 9 days before until 9 days after treatment application.

Days	April 2020 – June 2020		September 2020 – November 2020		December 2020 – February 2021	
	Control	**Biopesticide**	**Control**	**Biopesticide**	**Control**	**Biopesticide**
−9 – −7	296 ± 15	257 ± 22	354 ± 13	358 ± 27	338 ± 15	371 ± 29
−6 – −4	289 ± 22	272 ± 22	366 ± 15	376 ± 21	363 ± 18	366 ± 21
−3 – −1	246 ± 22	234 ± 28	362 ± 36	359 ± 14	379 ± 42	372 ± 22
0 – 2	304 ± 21	242 ± 26	379 ± 19	353 ± 15	376 ± 24	380 ± 27
3 – 5	248 ± 20	253 ± 37	385 ± 83	336 ± 24	372 ± 22	378 ± 20
6 – 8	234 ± 24	196 ± 16	346 ± 31	311 ± 20	364 ± 20	376 ± 27
p-value	0.061		0.27		0.52	

The values (±) accompanying the means are standard errors. Biopesticide is *Metarhizium anisopliae* ICIPE 69.

5.3.3. Conidial acquisition and persistence

Results of conidial acquisition and persistence are presented in Table 5.3. Conidia density significantly declined every three days after treatment applications on leaves ($p < 0.0001$), flowers ($p < 0.0001$) and bees ($p < 0.0001$). Based on Pearson product–moment correlation analysis, conidial acquisition by forager bees did not significantly affect pollen load ($R = 0.07$, $p = 0.44$).

Table 5.3: Biopesticide Colony-forming units (CFU) retained on the *Cucumis sativus* plant, and CFU and pollen collected by *Meliponula ferruginea* foragers.

*PTD	CFU/flower	CFU/leaf	CFU/bee	Pollen/bee
0	215,350 ± 226e	51,695,000 ± 9,000d	7,600 ± 54d	731 ± 74a
3	80,880 ± 613d	44,840,000 ± 9,000d	5,100 ± 67d	610 ± 74a
6	6,700 ± 193c	35,915,000 ± 13,000bc	1,080 ± 64c	581 ± 59a
9	1,600 ± 60b	25,635,000 ± 13,000bcd	780 ± 41bc	645 ± 44a
12	500 ± 50ab	14,225,000 ± 17,000abc	80 ± 0ab	607 ± 61a
15	200 ± 15ab	13,370,000 ± 9,000ab	20 ± 0a	687 ± 48a
18	0ab	7,190,000 ± 17,000a	0a	618 ± 55a

The values (±) accompanying the means are standard errors. Same small letters within the columns indicate no significant differences in the CFU and pollen load at $\alpha = 0.05$ according to the Tukey adjusted p-value probability. *PTD = post-treatment application days.

Conidial viability every three days for 18 days after treatment application is represented in Figure 5.8. Conidial viability significantly declined every three days after treatment application ($p < 0.0001$).

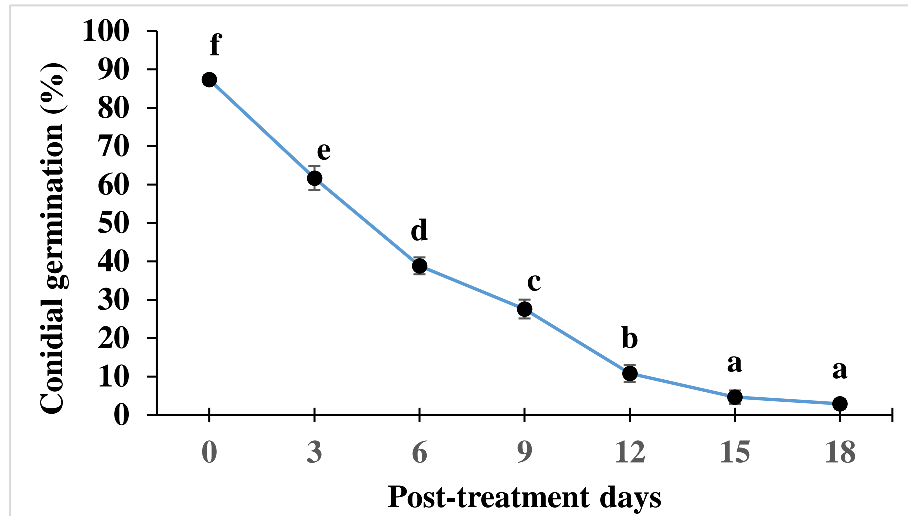


Figure 5.8: Conidial viability on *Cucumis sativus* after biopesticide spray.

Note: Error bars represent standard errors. Different letters above error bars are significantly different according to the Turkey test.

5.4. Discussion

This study shows that the *M. ferruginea* pollination behaviour such as flight activity, foraging activity, and pollen foraging and consequently cucumber yield (fruit set and fruit weight) was not affected by biopesticide (*Metarhizium anisopliae* ICIPE 69) spray.

Flight activity and foraging activity varied across experiments and the variations can be ascribed to several factors. Ferreira Junior *et al*. (2010) reported that variations in flight activity and floral resource collection activity of the stingless bee *Melipona bicolor schencki* Gribodo were effectuated by the environmental conditions and seasons. A similar trend is consistent with this study. Higher flight activity was recorded in April 2020 – June 2020 (first experiment) compared to September 2020 –

November 2020 (second experiment) and December 2020 – February 2021 (third experiment). On the other hand, foraging activity was higher in September 2020 – November 2020 than in April 2020 – June 2020 and December 2020 – February 2021. Higher foraging activity as recording here corresponding blooming periods in areas around the Kakamega forest where the *M. ferruginea* colonies were sourced from. This demonstrates *M. ferruginea* colony adjusts to floral resources. Also, the difference in foraging activity could be correlated to the number of flowers produced by a cucumber plant. The first, second and third experiments had 7.3 ± 2.0, 5.1 ± 1.2. and 7.9 ± 2.0 open flowers/plant/day and, therefore, the first and third experiments had low foraging activity than in the second experiment. On the contrary, flight activity did not exclusively reflect the blooming periods. Hive activity such as bees scouting for floral resources, collecting floral resources, removing waste out of the hive, and seeking water and propolis may have led to observed differential flight activity.

The observation days had a significant effect on flight activity and foraging activity and this can be associated with weather conditions, colony physiology, and plant phenology. For instance, the observation days corresponded to cucumber flowering stages (early, mid and late flowering) and the number of open flowers was highest at mid flowering followed by early flowering and least at late flowering stages.

This study provides the first overview of the effect of biopesticides on stingless bees under semi-field conditions. A study by Visalakshy *et al*. (2019) showed that *M. anisopliae* sprayed on mango *Mangifera indica* L. flowers did not significantly affect pollination activity of the dwarf honey bee *A. florea* Fabricius, the Asiatic honey bee *A. cerana* Fabricius and the hoverflies *Eristalis aryorum* Fain and *Chrysomya megacephala* Fabricius.

Forager bees may collect conidia alongside pollen from crops sprayed with fungal biopesticides. *Meliponula ferruginea* foragers collected the same amount of pollen (657 ± 29 pollen/bee) throughout the observation days. Similarly, these bees collected significantly similar numbers of conidia on day 0 (7,600 ± 54) and day 3 (5,100 ± 67) which then declined significantly on day 6 (1,080 ± 64), day 9 (780 ± 41), day 12 (80 ± 0), day 15 (20 ± 0) and day 18 (0 ± 0). Collected conidia did not affect the number of pollen collected nor did they reduce the *M. ferruginea* survival. Survival of forager bees ranged between 93 ± 3 – 84 ± 6% and 97 ± 2 – 88 ± 1% in biopesticide and control treatments, respectively. None of the bee cadavers developed mycosis, indicating that they are probably the nontarget insect group for tested biopesticide (*Metarhizium anisopliae* ICIPE 69).

The ability of biopesticides to persist in the environment is critical in providing extended protection against pests. But their persistence and viability may be affected by antifungal secretions by crops or environmental factors (Abbaszadeh *et al*., 2011). In this study, conidial persistence declined by 71.8% on flowers and 26.4% on leaves of the cucumber plant every three days. Equally, conidial viability declined by 41.9% every three days. This indicates that the efficacy of biopesticide may be reduced by the greenhouse's conditions especially by relatively high midday temperatures (28.7–31.6°C). This observation is consistent with Jaronski (2010) recording a decline in conidial viability of *B. bassiana* sprayed on melon *C. melo* L. plants with daily reduction of 9–11% and 47% on the underside and upper leaf surfaces, respectively.

The cucumber fruit set, fruit maturation, and fruit weight were significantly similar between biopesticide and control treatment. The fruit set was 91.1 ± 1.0% while fruit maturation was 84.8 ± 1.3%. However, in the second (357.1 ± 26.5g) and third

experiment (369.5 ± 22.3g), fruit weight was higher compared to the first experiment (255.9 ± 23.0g). Weather conditions and crop growing seasons could have affected the yield. April 2020 – June 2020 (first experiment) constitute the coldest months (17.6–19.3°C) with 1.9–4.9 mm precipitation and 67.0–72.0% RH while September 2020 – November 2020 (second experiment) and December 2020 – February 2021 (third experiment) are among the hottest months (18.3–19.3°C, 18.6–20.5°C) with 0.8–3.7 mm and 1.3–2.2 mm precipitation, and 58.0–75.0% and 53.0–70.0% RH, respectively, in Nairobi, Kenya (Merkel, 2019).

Based on this study, *M. anisopliae* ICIPE 69 does not affect *M. ferruginea* survival, pollination behaviour or cucumber yield and therefore can be used as a biopesticide in bee-pollinated crop systems as part of the IPPM component.

CHAPTER SIX

6.0. GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1. General conclusions

The application of entomopathogenic fungi (EPF) has been pragmatically proven effective in the management of several crop pests. However, when applied in pollinator-dependent crop systems, they might affect pollinators and the ecosystem services they provide. Bees are universally used as surrogate pollinators in most ecotoxicological studies. Understanding the effect of EPF on survival and pollination biology of bees, and the ability of EPF to thrive in bee colonies' conditions is important in the selection of EPF for the application in pollinator-resourced crop systems.

The objectives of this study were achieved and the findings showed that:

i. The bees acquired conidia when exposed to the EPF isolates. *Apis mellifera* acquired consistently higher conidia than *M. ferruginea*. Conidial acquisition by *A. mellifera* varied significantly among the isolates.
ii. Compared to the International Organization of Biological Control (IOBC) classification of biopesticides (Sterk *et al.*, 2000), the investigated EPF isolates are harmless or slightly harmful to bees. However, only three isolates of *M. anisopliae* (ICIPE 7, ICIPE 20, and ICIPE 69) had detectable negative effects on *A. mellifera* in the first experiment but not in the repeat experiment. The observed effects correlated strongly with conidial acquisition.
iii. The cardinal temperature models with inflection (CTMI) and Ratkowsky 3 conveniently described conidial germination and mycelial growth of all EPF isolates while generalized β function poorly described all these datasets. Brière

1, Brière 2, Ratkowsky 2, Lactin 1 and Van Der Heide poorly described most germination and growth datasets

iv. Temperature had a nonlinear effect on germination and growth of the isolates. Conidial germination for the isolates occurred between 13.2 to 36.3°C with the optima occurring between 26.2 to 28.9°C while mycelial growth occurred between 7.0 to 37.9°C with optima occurring between 25.9 to 28.4°C.

v. Under semi-field conditions, *M. anisopliae* ICIPE 69 sprayed on blooming cucumber plants did not affect *M. ferruginea* flight or foraging activity and cucumber fruit set and yield.

vi. Forager *M. ferruginea* collected conidia alongside pollen, however, the collected conidia did not affect pollen load or survival of forager bees.

vii. Conidia persisted on surfaces of blooming cucumber plants but their levels and viability declined gradually.

This study shows positive attributes of the tested *M. anisopliae* and *B. bassiana* isolates which include little to no effect on bees. However, as reported here, results from laboratory bioassays may not be conclusive considering that caged bees were secluded from the queen and exposed to artificial conditions. Caged bees, being eusocial insects, lacked well-coordinated functions that are arguably present in the hive colonies which include grooming and functional development. This may have increased the stress and noticeable susceptibility to the EPF isolates. In the semi-field conditions, EPF did not induce lethal or sublethal effects on bees. EPF germinated and grew maximally between 26.6 to 28.9°C and 25.9 to 28.4°C, respectively. This indicates that the isolates are unlikely to grow and affect the bees when intentionally or unintentionally introduced in the bee colonies.

6.2. Recommendations

This study recommends that:

1. The tested EPF are harmless to the bee pollinators and, thus, can be adopted as biopesticides to control arthropod pests in pollinator-resourced crop systems.
2. The CTMI and Ratkowsky 3 can be adopted to model the effect of temperature on EPF, especially during the selection and re-evaluation of the promising EPF.

Based on the findings of this study, future research should focus on:

1. Evaluating the effects of the EPF isolates on bees under *in situ* conditions where the bees are conditionally less stressful. For example, *M. anisopliae* ICIPE 7, *M. anisopliae* ICIPE 20, and *M. anisopliae* ICIPE 69 merit further investigations under *in situ* conditions.
2. Describing the growth performance of several other EPF candidates using mathematical modelling tools under different biophysical conditions found in the pest and nontarget insects' microhabitats.
3. Evaluating the presence of EPF conidia in the bee colony matrices including honey, pollen, broods and nursing bees.
4. Investigating the resistance mechanisms of stingless bees to promising EPF.

In conclusion, these tiered studies find fungal-based biopesticides developed in Africa to be harmless to bee pollinators. The investigated EPF can be safely used in IPM and IPPM programmes.

REFERENCES

Abbaszadeh, G., Dhillon, M. K., Srivastava, C. and Gautam, R. D. (2011). Effect of climatic factors on bioefficacy of biopesticides in arthropod pest management. *Biopesticides International*, *7*, 1–14.

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, *18*, 265–267.

Abou–Shaara, H. F. and Staron, M. (2019). Present and future perspectives of using biological control agents against pests of honey bees. *Egyptian Journal of Biological Pest Control*, *29*, 1–7.

Adams, M. P., Collier, C. J., Uthicke, S., Ow, Y. X., Langlois, L. and O'Brien, K. R. (2017). Model fit versus biological relevance: Evaluating photosynthesis–temperature models for three tropical seagrass species. *Scientific Reports*, *7*, 39930.

Adeoye, O. T., Pitan, O. R., Ademolu, K. O. and Ayandokun, A. E. (2020). Morphometric studies on Nigerian honey bee *Apis mellifera adansonni* L. workers of rainforest and Sudan agro-ecological zones of Nigeria. *International Journal of Tropical Insect Science*, *2020*, 1–9.

Akutse, K. S., Subramanian, S., Maniania, N. K. and Dubois, T. (2020). Biopesticide research and product development in Africa for sustainable agriculture and food security – Experiences from the International Centre of Insect Physiology and Ecology (*icipe*). *Frontiers in Sustainable Food Systems*, *4*, 563016.

Al Mazra'awi, M., Shipp, L., Broadbent, A. B. and Kevan, P. (2006). Dissemination of *Beauveria bassiana* by honey bees (Hymenoptera: Apidae) for control of tarnished plant bug (Hemiptera: Miridae) on canola. *Environmental Entomology*, *35*, 1569–1577.

Al Mazra'awi, M. (2007). Impact of the entomopathogenic fungus *Beauveria bassiana* on the honey bees, *Apis mellifera* (Hymenoptera: Apidae). *World Journal of Agricultural Sciences*, *3*, 7–11.

Al Toufailia, H., Alves, D. A., Bento, J. M. S., Marchini, L. C. and Ratnieks, F. L. W. (2016). Hygienic behaviour in Brazilian stingless bees. *Biology Open*, *5*, 1712–1718.

Alves, S. B., Marchini, L. C., Pereira, R. M. and Baumgratz, L. L. (1996). Effects of

some insect pathogens on the Africanized honey bee, *Apis mellifera* L. (Hym., Apidae). *Journal of Applied Entomology*, *120*, 559–564.

African Pollinator Initiative (API). (2004). *Browse and Pollinators in Africa: An Initial Stock–taking*. Food and Agriculture Organization, Rome, Italy.

Arena, M. and Sgolastra, F. (2014). A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology*, *23*, 324–334.

Asiko, G. A. (2012). Pollination of strawberry in Kenya, by stingless bees (Hymenoptera: Meliponini) and honey bee (Hymenoptera: Apini) for improved fruit quality [MSc Thesis, University of Nairobi, Kenya].

African Union – InterAfrican Bureau for Animal Resources (AU–IABAR). (2019). *The inaugural report on the status of apiculture in Africa 2019: Opportunities and strategies for development of the sector*. AU–IABAR, Nairobi, Kenya.

Badii, K. B., Billah, M. K. and Nyarko, G. (2015). Review of the pest status, economic impact and management of fruit–infesting flies (Diptera: Tephritidae) in Africa. *African Journal of Agricultural Research*, *10*(12), 1488–1498.

Bafo, W. (2019). Meliponiculture and physicochemical properties of honey produced by the African stingless bee *Plebeina hildebrandti* Friese in Kalakamati village, Botswana. *Botswana Journal of Agriculture and Applied Sciences*, *13*, 33–42.

Bahreini, R., Nasr, M., Docherty, C., de Herdt, O., Muirhead, S. and Feindel, D. (2020). Evaluation of potential miticide toxicity to *Varroa destructor* and honey bees, *Apis mellifera*, under laboratory conditions. *Scientific Reports*, *10*, 21529.

Bartomeus, I., Potts, S. G., Steffan–Dewenter, I., Vaissi`ere, B. E., Woyciechowski, M., Krewenka, K. M., Tscheulin, T., Roberts, S. P. M., Szentgy¨orgyi, H., Westphal, C. and Bommarco, R. (2014). Contribution of insect pollinators to crop yield and quality varies with agricultural intensification. *PeerJ*, *2*, 1–20.

Bassanezi R.B., Amorim L., Filho, A. B. and Hau, B. (1998). Effects of bean line pattern mosaic virus on the monocyclic components of rust and angular leaf spot of *Phaseolus* bean at different temperatures, *Plant Pathology*. 47, 289–298.

Bayissa, W., Ekesi, S., Mohamed, S. A., Kaaya, G. P., Wagacha, J. M., Hanna, R. and Maniania, N. K. (2017). Selection of fungal isolates for virulence against three aphid pest species of crucifers and okra. *Journal of Pest Science*, *90*, 355–368.

Baverstock, J., Roy, H. E. and Pell, J. K. (2010). Entomopathogenic fungi and insect behaviour: From unsuspecting hosts to targeted vectors. *The Ecology of Fungal*

Entomopathogens, *55*, 89–102.

Biesmeijer, J. C., Roberts, S. P. M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A. P., Potts, S. G., Kleukers, R., Thomas, C. D., Settele, J. and Kunin, W. E. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, *313*, 351–354.

Böhme, F., Bischoff, G., Zebitz, C. P. W., Rosenkranz, P. and Wallner, K. (2018). Pesticide residue survey of pollen loads collected by honey bees (*Apis mellifera*) in daily intervals at three agricultural sites in South Germany. *PLoS ONE*, *13*, 1–21.

Briere, J. F., Pracros, P., Le Roux, A. Y. and Pierre, J. S. (1999). A novel rate model of temperature-dependent development for arthropods. *Environmental Entomology*, *28*, 22–29.

Brittain, C. A., Vighi, M., Bommarco, R., Settele, J. and Potts, S. G. (2010). Impacts of a pesticide on pollinator species richness at different spatial scales. *Basic and Applied Ecology*, *11*, 106–115.

Bujok, B., Kleinhenz, M., Fuchs, S. and Tautz, J. (2002). Hot spots in the bee hive. *Naturwissenschaften*, *89*, 299–301.

Bull, J. C., Ryabov, E. V., Prince, G., Mead, A., Zhang, C., Baxter, L. A., Pell, J. K., Osborne, J. L. and Chandler, D. (2012). A strong immune response in young adult honey bees masks their increased susceptibility to infection compared to older bees. *PLoS Pathogens*, *8*, 11–14.

Butt, T. M., Ibrahim, L., Ball, B. V. and Clark, S. J. (1994). Pathogenicity of the entomogenous fungi *Metarhizium anisopliae* and *Beauveria bassiana* against crucifer pests and the honey bee. *Biocontrol Science and Technology*, *4*, 207–214.

Cappa, F., Petrocelli, I., Dani, F. R., Dapporto, L., Giovannini, M., Silva–Castellari, J., Turillazzi, S. and Cervo, R. (2019). Natural biocide disrupts nestmate recognition in honey bees. *Scientific Reports*, *9*, 1–10.

Carreck, N. L., Butt, T. M., Clark, S. J., Ibrahim, L., Isger, E. A., Pell, J. K. and Williams, I. H. (2006). Honey bees can disseminate a microbial control agent to more than one inflorescence pest of oilseed rape. *Biocontrol Science and Technology*, *17*, 179–191.

Celeste, M., Ferrari, F., Favaro, R., Mair, S., Zanotelli, L., Malagnini, V., Fontana, P. and Angeli, S. (2020). The application of *Metarhizium anisopliae* as a potential

biological control of *Varroa destructor* in Italy. *Journal of Apicultural Research*, *59*, 528–538.

Challa, G. K., Firake, D. M. and Behere, G. T. (2019). Bio–pesticide applications may impair the pollination services and survival of foragers of honey bee, *Apis cerana* Fabricius in oilseed brassica. *Environmental Pollution*, *249*, 598–609.

Chaplin–Kramer, R., Dombeck, E., Gerber, J., Knuth, K. A., Mueller, N. D., Mueller, M., Ziv, G. and Klein, A.–M. (2014). Global malnutrition overlaps with pollinator-dependent micronutrient production. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 1799–1799.

Chauzat, M.–P., Carpentier, P., Martel, A.–C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M. and Faucon, J.–P. (2009). Influence of pesticide residues on honey bee (Hymenoptera: Apidae) colony health in France. *Environmental Entomology*, *38*, 514–523.

Colombo, F. C., Maciel, R. M. A., Abati, R., Raulino–Domanski, F., Longhi, S. J., Costa–Maia, F. M., de Souza Vismara, E., Lozano, E. R. and Potrich, M. (2020). Do *Beauveria bassiana* and *Metarhizium anisopliae* affect worker survival and the production of Africanized *Apis mellifera* queens? *Journal of Apicultural Research*, *2020*, 1–10.

Conceição, P., Pereira, R. C., Souza, A. V., Sodré, G., Ribeiro, G., De Carvalho, C. A. and Neves, C. M. D. L. (2014). Susceptibility of *Melipona scutellaris* Latreille, 1811 (Hymenoptera: Apidae) worker bees to *Beauveria bassiana* (Bals.) Vuill. *Sociobiology*, *61*, 184–188.

Cuthbertson, A. G. S., Wakefield, M. E., Powell, M. E., Marris, G., Anderson, H., Budge, G. E., Mathers, J. J., Blackburn, L. F. and Brown, M. A. (2013). The small hive beetle *Aethina tumida*: A review of its biology and control measures. *Current Zoology*, *59*, 644–653.

Dantigny, P., Nanguy, S. P. M., Judet–Correia, D. and Bensoussan, M. (2011). A new model for germination of fungi. *International Journal of Food Microbiology*, *146*, 176–181.

Davidson, G., Phelps, K., Sunderland, K. D., Pell, J. K., Ball, B. V., Shaw, K. E. and Chandler, D. (2003). Study of temperature–growth interactions of entomopathogenic fungi with potential for control of *Varroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development. *Journal of Applied Microbiology*, *94*, 816–825.

De Faria, M. R. and Wraight, S. P. (2007). Mycoinsecticides and mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*, *43*, 237–256.

Delaplane, K. S., Van Der Steen, J. and Guzman–Novoa, E. (2013). Standard methods for estimating strength parameters of *Apis mellifera* colonies. *Journal of Apicultural Research*, *52*, 1–12.

Desneux, N., Decourtye, A. and Delpuech, J. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology*, *52*, 81–106.

Dimbi, S., Maniania, N. K. and Lux, S. A. (2004). Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl*, *49*, 83–94.

Dorigo, A. S., A. de S. Rosa–Fontana, H. M. Soares–Lima, J. S. Galaschi–Teixeira, R. C. F. Nocelli and O. Malaspina. (2019). *in vitro* larval rearing protocol for the stingless bee species *Melipona scutellaris* for toxicological studies. *PLoS One*. 14: e0213109.

Dorneles, A. L., De Souza Rosa, A. and Blochtein, B. (2017). Toxicity of organophosphorus pesticides to the stingless bees *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi*. *Apidologie*, *48*, 612–620.

Eardley, C. D. (2004). Taxonomic revision of the African stingless bees (Apoidea: Apidae: Apinae: Meliponini). *African Plant Protection*, *10*, 64–96.

Eardley, C. and Kwapong, P. (2013). Taxonomy as a tool for conservation of African stingless bees and their honey. In P. Vit *et al*. (Ed.), *Pot–Honey: A legacy of stingless bees*. Springer, New York, USA, 261–268.

Ekesi, S., Dimbi, S. and Maniania, N. K. (2007). The role of entomopathogenic fungi in the integrated management of fruit flies (Diptera: Tephritidae) with emphasis on species occurring in Africa. In E. Sunday and N. K. Maniania (Eds.), *Use of Entomopathogenic Fungi in Biological Pest Management*. Research Signpost, Kerala, India, 239–274.

Ekesi, S., Maniania, N. K. and Ampong–Nyarko, K. (1999). Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology*, *9*, 177–185.

Elzhov, T. V., Mullen, K. M., Spiess, A. –N. and Bolker, B. (2016). *minpack.lm*: R interface to the Levenberg–Marquardt nonlinear least-squares algorithm. R

package version 1.2–1. https://CRAN.R–project.org/package=minpack.lm

Espinosa–Ortiz, G. E., Lara–Reyna, J., Otero–Colina, G. and Valdez–Carrasco, R. A.–R. J. (2011). Susceptibility of larval, pupal and adult honey bees to isolates of *Beauveria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Sorokin) and *Paecilomyces fumosoroseus* (Wize). *Interciencia*, *36*, 148–152.

Fargues, J., Goettel, M. S., Smits, N., Ouedraogo, A. and Rougier, M. (1997). Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. *Mycologia*, *89*, 383–392.

Gallai, N., Salles, J., Settele, J. and Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics*, *68*, 810–821.

Gao, B. J., Mou, Y. N., Tong, S. M., Ying, S. H. and Feng, M. G. (2020). Subtilisin–like Pr1 proteases marking the evolution of pathogenicity in a wide–spectrum insect–pathogenic fungus. *Virulence*, *11*, 365–380.

Garibaldi, L. A., Steffan–Dewenter, I., Winfree, R., Aizen, A. M., Bommarco, R., Cunningham, S. A., Kremen, C., Carvalheiro, G. L., Harder, L. D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N. P., Dudenhöffer, H. J., Freitas, B. M., Ghazoul, J., Greenleaf, S., … Klein, A. M. (2013). Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*, *339*, 1608–1611.

Garibaldi, Lucas A., Aizen, M. A., Cunningham, S. and Klein, A. M. (2010). Pollinator shortage and global crop yield. *Communicative and Integrative Biology*, *2*, 37–39.

García–Fernández, P., Santiago–Álvarez, C. and Quesada–Moraga, E. (2008). Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of *Varroa* destructor (Acari: Mesostigmata), an ectoparasitic mite of honey bee, *Apis mellifera* (Hymenoptera: Apidae). *Apidologie*, *39*, 662–673.

Giannini, T. C., Cordeiro, G. D., Freitas, B. M., Saraiva, A. M. and Imperatriz–Fonseca, V. L. (2015). The dependence of crops for pollinators and the economic value of pollination in Brazil. *Journal of Economic Entomology*, *108*, 849–857.

Glare, T. R., Gwynn, R. L. and Moran–Diez, M. E. (2016). Development of biopesticides and future opportunities. *Methods in Molecular Biology*, *1477*, 211–221.

Gliñski, Z. and Buczek, K. (2003). Response of the Apoidea to fungal infections. *Apiacta*, *38*, 183–189.

Gougouli, M. and Koutsoumanis, K. P. (2012). Modeling germination of fungal spores at constant and fluctuating temperature conditions. *International Journal of Food Microbiology*, *152*, 153–161.

Gougouli, M. and Koutsoumanis, K. P. (2013). Relation between germination and mycelium growth of individual fungal spores. *International Journal of Food Microbiology*, *161*, 231–239.

Gougouli, M. and Koutsoumanis, K. P. (2010). Modelling growth of *Penicillium expansum* and *Aspergillus niger* at constant and fluctuating temperature conditions. *International Journal of Food Microbiology*, *140*(2–3), 254–262.

Goulnik, J., Plantureux, S., Van Reeth, C., Baude, M., Mesbahi, G. and Michelot–Antalik, A. (2020). Facial area and hairiness of pollinators visiting semi-natural grassland wild plants predict their facial pollen load. *Ecological Entomology*, *2020*, 1–11.

Graves, S., Piepho, H.–P. and Selzer, L. (2019). *multcompView:* Visualizations of paired comparisons. R package version 0.1–8. https://cran.r–project.org/web/packages/multcompView/index.html

Greenfield, B. P. J., Lord, A. M., Dudley, E. and Butt, T. M. (2014). Conidia of the insect pathogenic fungus, *Metarhizium anisopliae*, fail to adhere to mosquito larval cuticle. *Royal Society Open Science*, *1*, 140193–140193.

Hamiduzzaman, M. M., Sinia, A., Guzman–Novoa, E. and Goodwin, P. H. (2012). Entomopathogenic fungi as potential biocontrol agents of the ecto–parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.). *Journal of Invertebrate Pathology*, *111*, 237–243.

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J. F., Aupinel, P., Aptel, J., Tchamitchian, S. and Decourtye, A. (2012). A common pesticide decreases foraging success and survival in honey bees. *Science*, *336*, 348–350.

Hlina, B. L. (2020). *ecotox:* A package for analysis of ecotoxicology. R package version 1.4.2. https://cran.r–project.org/package=ecotox

Hokkanen, H. M. T., Zeng, Q. Q. and Menzler–Hokkanen, I. (2003). Assessing the impacts of *Metarhizium* and *Beauveria* on bumblebees. In H.M.T. Hokkanen and A.E. Hajek (Eds.), *Environmental Impacts of Microbial Insecticides.* Springer, Dordrecht, Netherlands, 63–71.

Hothorn, T., Bretz, F. and Westfall, P. (2008). *multicomp*: Simultaneous inference in general parametric models. R package version 1.4–14. *Biometrical Journal*, *50*, 346–363.

Huang, L., Hwang, C.–A. and Phillips, J. (2011). Evaluating the effect of temperature on microbial growth rate–The Ratkowsky and a Bělehrádek–type models. *Journal of Food Science*, *76*, 547–557.

Ibrahim, A. A., Mohamed, H. F., El–Naggar, S. E. M., Swelim, M. A. and Elkhawaga, O. E. (2016). Isolation and selection of entomopathogenic fungi as biocontrol agent against the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae). *Egyptian Journal of Biological Pest Control*, *26*, 249–253.

IPBES (2016). The assessment report of the Intergovernmental Science–Policy Platform on Biodiversity and Ecosystem Services (IPBES) on pollinators, pollination and food production. S.G. Potts, V. L. Imperatriz–Fonseca, and H. T. Ngo, (Eds). IPBES Secretariat, Bonn, Germany. 552.

Jarimi, H., Tapia–Brito, E. and Riff, S. (2020). A review on thermoregulation techniques in honey bees' (*Apis mellifera*) beehive microclimate and its similarities to the heating and cooling management in buildings. *Future Cities and Environment*, *6*, 1–8.

Jaronski, S. T. (2010). Ecological factors in the inundative use of fungal entomopathogens. *BioControl*, *55*, 159–185.

Jones, J. C., Myerscough, M. R., Graham, S. and Oldroyd, B. P. (2004). Honey bee nest thermoregulation: Diversity promotes stability. *Science*, *305*, 402–404.

Jones, J. C., Nanork, P. and Oldroyd, B. P. (2007). The role of genetic diversity in nest cooling in a wild honey bee, *Apis florea. Journal of Comparative Physiology A, 193*, 159–165.

Jones, J. C. and Oldroyd, B. P. (2006). Nest thermoregulation in social insects. *Advances in Insect Physiology*, *33*, 153–191.

Kajobe, R. (2006). Pollen foraging by *Apis mellifera* and stingless bees Meliponula bocandei and *Meliponula nebulata* in Bwindi Impenetrable National Park, Uganda. *African Journal of Ecology*, *45*, 265–274.

Kambura, C., Tanga, C. M., Kilalo, D., Muthomi, J., Salifu, D., Rwomushana, I., Mohamed, S. A. and Ekesi, S. (2018). Composition, host range and host suitability of vegetable–infesting Tephritids on cucurbits cultivated in Kenya.

African Entomology, *26*, 379–397.

Kanga, L. H.B., James, R. R. and Boucias, D. G. (2002). *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential microbial control agents of *Varroa destructor*, a honey bee parasite. *Journal of Invertebrate Pathology*, *81*, 175–184.

Kanga, Lambert H. B., Jones, W. A. and James, R. R. (2009). Field trials using the fungal pathogen, *Metarhizium anisopliae* (Deuteromycetes: Hyphomycetes) to control the ectoparasitic mite, *Varroa destructor* (Acari: Varroidae) in honey bee, *Apis mellifera* (Hymenoptera: Apidae) colonies. *Journal of Economic Entomology*, *96*, 1091–1099.

Kapongo, J. P., Shipp, L., Kevan, P. and Broadbent, B. (2008). Optimal concentration of *Beauveria bassiana* vectored by bumblebees in relation to pest and bee mortality in greenhouse tomato and sweet pepper. *Biological Control*, *53*, 797–812.

Kasina, J. M., Mburu, J. and Kraemer, M. (2009). Economic benefit of crop pollination by bees: a case of Kakamega small-holder farming in western Kenya. *Journal of Economic Entomology*, *202*, 467–473.

Kassambara, A., Kosinski, M. and Biecek, P. (2020). *survminer*: A package for drawing survival curves using “*ggplot2*”. R package version 0.4.8. https://cran.r–project.org/package=survminer

Kevan, P., Kapongo, J.–P., Al Mazra’awi, M. S. and Shipp, L. (2008). Honey bees, bumblebees, and biocontrol. In R. R. James and T. L. Pitts–Singer (Eds.), *Bee Pollination in Agricultural Ecosystems*. Oxford University Press, Oxford, England, 65–75

Kiatoko, N., Raina, S. K., Muli, E. and Mueke, J. (2014). Enhancement of fruit quality in *Capsicum annum* through pollination by *Hypotrigona gribodoi* in Kakamega, Western Kenya. *Entomological Science, 17*, 106–110.

Kibira, M.,Affognon, H., Njehia, B., Muriithi, B., Mohamed, S. and Ekesi, S. (2010). Economic evaluation of integrated management of fruit fly in mango production in Embu County, Kenya. *Africa Journal of Agricultural and Resource Economics*, *10*(4), 343-353.

Kidanu, S. and Hagos, L. (2020). Research and the application of entomopathogenic fungi as pest management option: A review. *Journal of Environment and Earth Science*, *10*, 32–39.

Kivett, A. J. M., Cloyd, R. A. and Bello, N. M. (2016). Evaluation of entomopathogenic fungi against the western flower thrips (Thysanoptera: Thripidae) under laboratory conditions. *Journal of Entomological Sciences*, *51*, 274–291.

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan–Dewenter, I., Cunningham, S. A., Kremen, C. and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceeding of the Royal Society B*, *274*, 303–313.

Kumar, S., Joshi, P. C., Nath, P. and Singh, V. K. (2018). Impacts of insecticides on pollinators of different food plants. *Entomology, Ornithology and Herpetology*, *7*, 1–6.

Kvålseth, T. O. (1985). Cautionary note about R^2. *American Statistician*, *39*, 279–285.

Lactin, D. J., Holliday, N. J., Johnson, D. L. and Craigen, R. (1995). Improved rate model of temperature-dependent development by arthropods. *Environmental Entomology*, *24*, 68–75.

Lenth, R. V. (2015). *lsmeans*: Least–squares means. R package version 2.30–0. *Journal of Statistical Software*, *69*, 1–33.

Liu, H., Skinner, M., Brownbridge, M. and Parker, B. L. (2003). Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae). *Journal of Invertebrate Pathology*, *82*, 139–147.

Maina, U. M., Galadima, I. B., Gambo, F. M. and Zakaria, D. (2018). A review on the use of entomopathogenic fungi in the management of arthropod pests of field crops. *Journal of Entomology and Zoology Studies*, *6*, 27–32.

Malan, A. P., Von Diest, J. I., Moore, S. D. and Addison, P. (2018). Control options for false codling moth, *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), in South Africa, with emphasis on the potential use of entomopathogenic nematodes and fungi. *African Entomology*, *26*, 14–29.

Mangiafico, S. (2020). *rcompanion:* Functions to support extension education program evaluation. R package version 2.3.25. https://CRAN.R–project.org/package=rcompanion

Martins, D. (2014). Our friends the pollinators: A handbook of pollinator diversity and conservation in East Africa. In *The East Africa Natural History Society*. Nature, Nairobi, Kenya, 1–102.

Medrzycki, P., Giffard, H., Aupinel, P., Belzunces, L. P., Chauzat, M. P., Claßen, C., Colin, M. E., Dupont, T., Girolami, V., Johnson, R., Conte, Y. Le, Lückmann, J., Marzaro, M., Pistorius, J., Porrini, C., Schur, A., Sgolastra, F., Delso, N. S., Van Der Steen, J. J. M., … Vidau, C. (2013). Standard methods for toxicology research in *Apis mellifera*. *Journal of Apicultural Research*, *52*, 1–61.

Medrzycki, P., Sgolastra, F., Bortolotti, L., Bogo, G., Tosi, S., Padovani, E., Porrini, C. and Sabatini, A. G. (2010). Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides. *Journal of Apicultural Research*, *49*, 52–59.

Meikle, W.G, Mercadier, G., Holst, N., Nansen, C., Meikle, W. G., Mercadier, G., Holst, N., Nansen, C. and Girod, V. (2008). Impact of a treatment of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) on honey bee (*Apis mellifera*) colony health and on *Varroa destructor* mites (Acari: Varroidae). *Apidologie*, *39*, 247–259.

Meikle, W G, Mercadier, G., Annas, F., Holst, N., Meikle, W. G., Mercadier, G., Annas, F. and Holst, N. (2009). Effects of multiple applications of a *Beauveria* based biopesticide on *Varroa destructor* (Acari: Varroidae) densities in honey bee (Hymenoptera: Apidae) colonies. *Journal of Apicultural Research and Bee World*, *48*, 220–222.

Meikle, William G., Adamczyk, J. J., Weiss, M. and Gregorc, A. (2018). Effects of bee density and sublethal imidacloprid exposure on cluster temperatures of caged honey bees. *Apidologie*, *49*, 581–593.

Meikle, William G., Weiss, M., Maes, P. W., Fitz, W., Snyder, L. A., Sheehan, T., Mott, B. M. and Anderson, K. E. (2017). Internal hive temperature as a means of monitoring honey bee colony health in a migratory beekeeping operation before and during winter. *Apidologie*, *48*, 666–680.

Merkel, A. (2019). Nairobi climate. AM online projects climate–data.org. https://en.climate–data.org/africa/kenya/nairobi/nairobi–541/. Accessed 14 May 2021

Merkle, E. and You, D. (2020). *nonnest2*: Tests of non–nested models. R package version 0.5–5. https://CRAN.R–project.org/package=nonnest2

Miętkiewski, R., Tkaczuk, C., Żurek, M. and Van der Geest, L. P. (2014). Temperature requirements of four entomopathogenic fungi. *Acta Mycologica*, *29*, 109–120.

Mommaerts, V. and Smagghe, G. (2011a). Side–effects of pesticides on the pollinator *Bombus*: An overview. In *Pesticides in the Modern World–Pests Control and Pesticides Exposure and Toxicity Assessment*. IntechOpen, Rijeka, Croatia, 507–552.

Mommaerts, V. and Smagghe, G. (2011b). Entomovectoring in plant protection. *Arthropod–Plant Interactions*, *5*, 81–95.

Mommaerts, V., Sterk, G. and Smagghe, G. (2009). A laboratory evaluation to determine the compatibility of microbiological control agents with the pollinator *Bombus terrestris*. *Pest Management Science*, *65*, 949–955.

Mora, M. A. E., Castilho, C. A. and Fraga, M. E. (2017). Classification and infection mechanism of entomopathogenic fungi: Review article. *Arquivos do Instituto Biológico*, *84*, 1–10.

Muerrle, T. M., Neumann, P., Dames, J. F., Hepburn, H. R. and Hill, M. P. (2007). Susceptibility of adult *Aethina tumida* (Coleoptera: Nitidulidae) to entomopathogenic fungi. *Journal of Economic Entomology*, *99*, 1–6.

Muli, E., Patch, H., Frazier, M., Frazier, J., Torto, B., Baumgarten, T., Kilonzo, J., Ng, J., Mumoki, F., Masiga, D., Tumlinson, J. and Grozinger, C. (2014). Evaluation of the distribution and impacts of parasites, pathogens, and pesticides on honey bee (*Apis mellifera*) populations in East Africa. *PLoS ONE*, *9*, e94459.

Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., Van Engelsdorp, D. and Pettis, J. S. (2010). High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. *PLoS ONE*, *5*, e9754.

Namusana, H. and Emiru, S. (2010). Evaluation of native fungal isolates of *Metarhizium anisopliae* var. *acridum* and *Beauveria bassiana* against the greater wax moth, *Galleria mellonella* L. (Pyralidae: Lepidoptera) in Ethiopia. *Ethiopian Journal of Science*, *33*, 117–124.

Niassy, S, Maniania, N. K., Subramanian, S., Gitonga, L. M., Mburu, D. M., Masiga, D. and Ekesi, S. (2012). Selection of promising fungal biological control agent of the western flower thrips *Frankliniella occidentalis* (Pergande). *Letters in Applied Microbiology*, *54*, 487–493.

Niassy, S., Subramanian, S., Ekesi, S., Bargul, J. L., Villinger, J. and Maniania, N. K. (2013). Use of *Metarhizium anisopliae* chitinase genes for genotyping and virulence characterization. *BioMed Research International*, *2013*, 1–9.

Nkoba, K., Raina, S. K., Muli, E. and Mitho, K. (2012). Species richness and nest dispersion of some tropical meliponine bees (Apidae: Meliponinae) in six habitat types in the Kakamega forest, western Kenya. *International Journal of Tropical Insect Science*, *32*, 194–202.

Nur, S., Demirci, Ş. and Altuntaş, H. (2019). Entomopathogenic potential of *Purpureocillium lilacinum* against the model insect *Galleria mellonella* (Lepidoptera: Pyralidae). *Environmental and Experimental Biology*, *17*, 71–74.

Odanga, J., Olubayo, F., Nyankanga, R. and Mwalusepo, S. (2017). Records of arthropod species sampled from avocado plant (*Persea americana* Mill) in small–scale agro–ecosystems at Taita Hills and Mount Kilimanjaro. *International Journal of Environment, Agriculture and Biotechnology (IJEAB)*, *2*, 2457–2465.

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

Ostiguy, N., Drummond, F. A., Aronstein, K., Eitzer, B., Ellis, J. D., Spivak, M. and Sheppard, W. S. (2019). Honey bee exposure to pesticides: A four–year nationwide study. *Insects*, *10*, 1–34.

Pasupuleti, V. R., Sammugam, L., Ramesh, N. and Gan, S. H. (2017). Honey, propolis, and royal jelly: A comprehensive review of their biological actions and health benefits. *Oxidative Medicine and Cellular Longevity*, *2017*, 1–21.

Peleg, M. and Normand, M. D. (2013). Modeling of fungal and bacterial spore germination under static and dynamic conditions. *Applied and Environmental Microbiology*, *79*, 6764–6775.

Pohorecka, K., Skubida, P., Semkiw, P., Miszczak, A., Teper, D., Sikorski, P., Zagibajlo, K., Skubida, M., Zdańska, D. and Bober, A. (2013). Effects of exposure of honey bee colonies to neonicotinoid seed–treated maize crops. *Journal of Apicultural Science*, *57*, 199–208.

Porto, R. G., De Almeida, R. F., Cruz–Neto, O., Tabarelli, M., Viana, B. F., Peres, C. A. and Lopes, A. V. (2020). Pollination ecosystem services: A comprehensive review of economic values, research funding and policy actions. *Food Security*, *12*, 1425–1442.

Potrich, M., da Silva, R. T. L., Maia, F. M. C., Lozano, E. R., Rossi, R. M., Colombo, F. C., Tedesco, F. G. and de Gouvea, A. (2018). Effect of entomopathogens on Africanized *Apis mellifera* L. (Hymenoptera: Apidae).

Revista Brasileira de Entomologia, *62*, 23–28.

Potts, S. G, Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W. E. (2010). Global pollinator declines: Trends, impacts and drivers. *Trends in Ecology and Evolution*, *25*, 345–353.

Potts, S. G., Imperatriz–fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C. and Breeze, T. D. (2016). Safeguarding pollinators and their values to human well–being. *Nature*, *540*, 220–229.

Quesada–Moraga, E., Maranhao, E. A. A., Valverde–García, P. and Santiago–Álvarez, C. (2006). Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity. *Biological Control, 36*, 274–287.

R Core Team. (2020). R: A language and environment for statistical computing. R software version 4.0.3. R Foundation for Statistical Computing, Vienna, Austria. https://www.R–project.org/

Rangel, D. E. N., Braga, G. U. L., Anderson, A. J. and Roberts, D. W. (2005). Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographic origins. *Journal of Invertebrate Pathology*, *88*, 116–125.

Ratkowsky, D. A., Lowry, R. K., McMeekin, T. A., Stokes, A. N. and Chandler, R. E. (1983). Model for bacterial culture growth rate throughout the entire biokinetic temperature range. *Journal of Bacteriology*, *154*, 1222–1226.

Rodríguez, M., Gerding, M., France, A. and Ceballos, R. (2009). Evaluation of *Metarhizium anisopliae* var. *anisopliae* Qu–M845 isolate to control *Varroa destructor* (Acari: Varroidae) in laboratory and field trials. *Chilean Journal Of Agricultural Research*, *69*, 541–547.

Rosso, L., Lobry, J. R., Bajard, S. and Flandrois, J. P. (1995). Convenient model to describe the combined effects of temperature and pH on microbial growth. *Applied and Environmental Microbiology*, *61*, 610–616.

Roubik, D. W. (2006). Stingless bee nesting biology. *Apidologie*, *37*, 124–143.

Sandhu, S. S., Sharma, A. K., Beniwal, V., Goel, G., Batra, P., Kumar, A., Jaglan, S., Sharma, A. K. and Malhotra, S. (2012). Myco–biocontrol of arthropod pests: Factors involved, mechanism, and regulation. *Journal of Pathogens*, *2012*, 10.

Shahid, A. A. L. I., Rao, A. Q., Bakhsh, A. and Husnain, T. (2012). Entomopathogenic fungi as biological controllers: New insights into their

virulence and pathogenicity. *Archive of Biological Sciences*, *64*, 21–42.

Sharma, A., Rana, C. and Shiwani, K. (2016). Important arthropod pests of cucurbits and their management. In M. Pessarakli (Ed.), *Handbook of Cucurbits: Growth, Cultural Practices, and Physiology*. CRC Press, Florida, USA, 328–351

Shipp, L., Pierre, J., Park, H. and Kevan, P. (2012). Effect of bee–vectored *Beauveria bassiana* on greenhouse beneficials under greenhouse cage conditions. *Biological Control*, *63*, 135–142.

Singh, S., Singh, A. P., Chaurasiya, D. K., Mukherjee, S. and Mondal, G. (2020). Entomopathogenic fungi as biocontrol agents in agriculture. *Research Today*, *2*, 264–266.

Sinia, A. and Guzman–novoa, E. (2018). Evaluation of the entomopathogenic fungi *Beauveria bassiana* GHA and *Metarhizium anisopliae* UAMH 9198 alone or in combination with thymol for the control of *Varroa destructor* in honey bee (*Apis mellifera*) colonies. *Journal of Apicultural Research*, *57*, 308–316.

Slaa, E. J., Chaves, L. A. S., Malagodi–Bragac, K. S. and Hofsteded, F. E. (2006). Stingless bees in applied pollination: Practice and perspectives. *Apidologie*, *37*, 293–315.

Smagghe, G. U., Meyer, L. De, Meeus, I. and Mommaerts, V. (2013). Safety and acquisition potential of *Metarhizium anisopliae* in entomovectoring with bumblebees, *Bombus terrestris*. *Journal of Economic Entomology*, *106*, 11–16.

Smits, N., Brière, J. F. and Fargues, J. (2003). Comparison of non–linear temperature-dependent development rate models applied to *in vitro* growth of entomopathogenic fungi. *Mycological Research*, *107*, 1476–1484.

Soni, J. and Thakur, M. (2011). Effect of biopathogens on honey bees. *Pest Technology*, *5*, 86–90.

Souza, B. A., Roubik, D. W., Barth, O. M., Heard, T. A., Enríquez, E., Carvalho, C., Villas–Bôas, J., Marchini, L., Locatelli, J. C., Persano–Oddo, L., Almeida–Muradian, L., Bogdanov, S. and Patricia, V. (2006). Composition of stingless bee honey: Setting quality standards. *Interciencia*, *31*, 867–875.

Spivak, M., Masterman, R., Ross, R. and Mesce, K. A. (2003). Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *Journal of Neurobiology*, *55*, 341–354.

Stabentheiner, A. (2001). Thermoregulation of dancing bees: Thoracic temperature of pollen and nectar foragers in relation to profitability of foraging and colony need.

Journal of Insect Physiology, *47*(4–5), 385–392.

Sterk, G., Heuts, F., Merck, N., Bock, J. and Biobest, N. V. (2000). Sensitivity of nontarget arthropods and beneficial fungal species to chemical and biological plant protection products: Results of laboratory and semi-field trials. *1st International Symposium on Biological Control of Arthropods*, 306–313.

Tefera, T. and Pringle, K. (2003). Germination, radial growth, and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* isolates and their virulence to *Chilo partellus* (Lepidoptera: Pyralidae) at different temperatures. *Biocontrol Science and Technology*, *13*, 699–704.

Therneau, T. M. and Lumley, T. (2020). *survival*: Survival analysis. R package version 3.2–3. https://CRAN.R–project.org/package=survival

Toledo–Hernandez, R. A., Ruiz–Toledo, J., Toledo, J. and Sanchez, D. (2016). Effect of three entomopathogenic fungi on three species of stingless bees (Hymenoptera: Apidae) under laboratory conditions. *Journal of Economic Entomology*, *109*(3), 1015–1019.

Vanbergen, A., Heard, M., Breeze, T., Potts, S. and Hanley, H. (2014). Status and value of pollinators and pollination services. A report to the Department for Environment, Food and Rural Affairs (Defra), London, UK.

Vanbergen, A. J., Garratt, M. P., Vanbergen, A. J., Baude, M., Biesmeijer, J. C., Britton, N. F., Brown, M. J. F., Brown, M., Bryden, J., Budge, G. E., Bull, J. C., Carvell, C., Challinor, A. J., Connolly, C. N., Evans, D. J., Feil, E. J., Garratt, M. P., Greco, M. K., Heard, M. S., … Wright, G. A. (2013). Threats to an ecosystem service: Pressures on pollinators. *Frontiers in Ecology and the Environment*, *11*(5), 251–259.

Van Der Heide, T., Roijackers, R. M. M., Nes, E. H. Van, and Peeters, E. T. H. M. (2006). A simple *Eqn* for describing the temperature dependent growth of free–floating macrophytes. *Aquatic Botany*, *84*, 171–175.

Vandervalk, L. P., Nasr, M. E. and Dosdall, L. M. (2014). New miticides for integrated pest management of *Varroa destructor* (Acari: Varroidae) in honey bee colonies on the Canadian Prairies. *Journal of Economic Entomology*, *107*(6), 2030–2036.

Visalakshy, P. N. G., Darshana, C., Lewis, F., Swathi, C. and Reddy, P. V. R. (2019). Safety of an oil based formulation of entomopathogen, *Metarhizium anisopliae* to pollinators of mango. *Pest Management in Horticultural System*, *25*(2), 186–

189.

Vuong, Q., (1989). Likelihood ratio tests for model selection and non–nested hypotheses. *Econometrica*, *57*(2), 30–333.

Wanchoo, A., Lewis, M. W. and Keyhani, N. O. (2009). Lectin mapping reveals stage–specific display of surface carbohydrates in *in vitro* and haemolymph–derived cells of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology*, *155*, 3121–3133.

Warra, A. A. and Prasad, M. N. V. (2020). African perspective of chemical usage in agriculture and horticulture—their impact on human health and environment. In M. N. V. Prasad (Ed.), *Agrochemicals Detection, Treatment and Remediation*. Butterworth–Heinemann Elsevier, Oxford, United Kingdom. 401–436.

Wiest, L., Buleté, A., Giroud, B., Fratta, C., Amic, S., Lambert, O., Pouliquen, H. and Arnaudguilhem, C. (2011). Multi–residue analysis of 80 environmental contaminants in honeys, honey bees and pollen by one extraction procedure followed by liquid and gas chromatography coupled with mass spectrometric detection. *Journal of Chromatography A*, *1218*, 5743–5756.

Woodcock, B. A., Bullock, J. M., Shore, R. F., Heard, M. S., Pereira, M. G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S. and Pywell, R. F. (2017). Country–specific effects of neonicotinoid pesticides on honey bees and wild bees. *Science*, *356*, 1393–1395.

Zimmermann, G. (2007). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology*, *17*, 553–596.

Zwietering, M. H., De Koos, J. T., Hasenack, B. E., De Wit, J. C. and Van ’T Riet, K. (1991). Modeling of bacterial growth as a function of temperature. *Applied and Environmental Microbiology*, *57*, 1094–1101.