LABORATORY AND FIELD EVALUATION OF ENTOMOPATHOGENIC FUNGI, *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA*, FOR MANAGEMENT OF THE LEGUME POD BORER, *MARUCA VITRATA* (FABRICIUS) ON COWPEA

BY

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DECLARATION AND APPROVAL

Declaration by the candidate

I, **Venansio Tumuhaise** (Registration Number: **I80/81921/2011)** declare that this thesis is my original work and, to the best of my knowledge, it has not been presented for the award of a degree in any other University.

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DEDICATION

To my beloved wife Jennifer and our children, Ryan and Louis;
Your prayers, support, perseverance and encouragement kept me strong and determined; you were honest partners in this struggle,
which finally comes to meaningful conclusion.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

APU	Arthropod Pathology Unit
ANOVA	Analysis Of Variance
ARPPIS	African Regional Postgraduate Programme in Insect Science
BMZ	German Federal Ministry for Economic Cooperation and Development
CFU	Colony Forming Units
DAAD	German Academic Exchange Service
DAE	Days After Emergence
DAT	Days After Treatment
EPF	Entomopathogenic Fungi
GIS	Geographic Information System
HSD	Honestly Significance Difference
ICIPE/*icipe*	International Centre of Insect Physiology and Ecology
ILCYM	Insect Life Cycle Modeling
IPM	Integrated pest management
LC_{50}	Concentration at which 50% mortality of the population occurred
LPB	Legume Pod Borer
LT_{50}	Time at which 50% mortality of the population occurred
RH	Relative Humidity
SDA	Saboraud Dextrose Agar
SE	Standard Error
SSA	Sub-Saharan Africa
USA	United States of America
UV	Ultra Violet

ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important food and forage legumes in the semi-arid tropics. The legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) is considered as one of the most important insect pests constraining cowpea production, causing yield losses of 20 – 80% across different parts of the world. Use of chemical pesticides is the most widely known form of pest control on cowpea. Exploitation of microbial pesticides has been gaining increased attention and interest among those concerned with developing environmentally friendly and safe approaches and tactics for pest management. This study aimed at evaluating the potential of entomopathogenic fungi (EPF) for the management of *M. vitrata* on cowpea. Screening of EPF was done to identify the most potent isolates against *M. vitrata*. Fourteen isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin and six of *Beauveria bassiana* (Balsamo) Vuillemin were screened against first instar larvae, from which the best two isolates namely *M. anisopliae* ICIPE 18 and ICIPE 69 that caused highest mortality of 91% and 81%, respectively were selected for further studies. Assessment of biomass and propagule production in four liquid media showed that isolate ICIPE 69 outperformed ICIPE 18, while Jenkins-Prior and APU1 ranked as best media. Thermotolerance studies revealed that 25 ^{o}C and 30^{o}C were optimal temperature for germination for ICIPE 18 and ICIPE 69, respectively, while optimal temperature for growth were 30^{o}C and 33^{o}C for ICIPE 69 and ICIPE 18, respectively. All isolates were virulent against *M. vitrata* at all temperatures causing mortality of 56.0 – 91.6% across all temperatures (15^{o}C – 33^{o}C). Host plant effects were detected whereby insects raised on cowpea had the highest mortality due to isolate ICIPE 18 (77.5%) while those reared on bean had the lowest mortality (36.6%). On the other hand, virulence of ICIPE 69 was highest on beans (95.7%), and lowest on *Cajanus cajan*. *Cajanus cajan* induced significantly fewer colony forming units (CFU) for ICIPE 69. Fungal persistence was affected by plant, fungal isolate, and time factors resulting in more than 90% reduction in CFU at 3 days post spraying. Field evaluation results showed that Karate®, the commercial formulation of *M.*

anisopliae ICIPE 69 (Campaign®), and Nimbecidine®, significantly reduced pest damage which translated into grain yield increment of up to 1254 Kg/ha (387%), 747kgs/ha (231%), and 340 kg/ha (117%), respectively. This translated into marginal returns of up to 5.7 (Karate®), 3.1 (Campaign®) and 1.2 (Nimbecidine®). Overall, the study demonstrated that *M. anisopliae* isolates ICIPE 18 and ICIPE 69 are effective against *M. vitrata*, and that isolate ICIPE 69 produced more biomass and propagules than ICIPE 18, in Jenkins-Prior and APU1 liquid media. Temperature and host plant factors affect the virulence of isolates ICIPE 18 and ICIPE 69. Overall, the commercial formulation of *M. anisopliae* isolate ICIPE 69 (Campaign®) which was effective against *M. vitrata* in field trials is hereby recommended as an effective biopesticide for protecting cowpea against the insect pest. Incorporating Camapaign into IPM package for cowpea would not only protect the crop against pest damage but also minimize risks associated with chemical pesticides.

Key words: *Maruca vitrata*; *Metarhizium anisopliae*; *Beauveria bassiana*; temperature; *Vigna unguiculata*; *Cajanus cajan*; *Phaseolus vulgaris*; nimbecidine®; Campaign®; Lambda-cyhalothrin; marginal returns.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Cowpea (*Vigna unguiculata* L. Walp) is one of the most important food and forage legumes in the semi-arid tropics that includes parts of Asia, Africa, Southern Europe, Southern United States, and Central and South America (Singh, 2005; Adati *et al.*, 2008; Timko and Singh, 2008; Dugje *et al.*, 2009). Cowpea seeds are a major source of plant proteins and vitamins for human, animal feed, and also serve as a major source of income. The crop also plays an important role in providing soil nitrogen especially in areas with poor soil fertility (Singh, 2005). The ability of cowpea to thrive in hot and dry conditions further makes it an important crop that could contribute towards mitigating effects of climate change especially in sub-Saharan Africa (SSA) (Singh, 2005; Adati *et al.*, 2008; Timko and Singh, 2008; Dugje *et al.*, 2009).

Cowpea production is hampered by a number of abiotic and biotic constraints including diseases, insect pests, and parasitic plants (Singh and Allen, 1979; Singh and Emden, 1979; Jackai and Daoust, 1986; Jackai and Adalla, 1997). Drought, low soil fertility, and high salinity are some of the abiotic factors limiting cowpea production (Timko and Singh, 2008).Of the biotic factors, insect pests are considered the major constraint to cowpea production, and the most damaging are those that occur during the reproductive stages which include flower thrips, pod suckers and the pod borers (Singh and Allen, 1979; Singh and Emden, 1979; Jackai and Daoust, 1986; Sureja *et al.*, 2010). The legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) is considered as one of the most important pests of cowpea and other leguminous crops in SSA and other parts of the tropics (Karel, 1985; Sharma, 1998; Sharma *et al.*, 1999). Damage is caused by the larvae which feed mainly on floral buds, flowers and pods. A single larva can consume 4 – 6

flowers before larval development is completed (Sharma, 1998). Cowpea yield losses of 20–80% have been reported in SSA due to this pest (Okeyo-Owuor *et al.*, 1983; Karel, 1985; Sharma, 1998). The pest is also reported to cause 20–30% yield loss in mungbean and yardlong bean in Asia (Sharma, 1998).

Management of *M. vitrata* has relied largely on the use of insecticides (Agwu, 1997; Tamò *et al.*, 1997; Sharma, 1998). Misue of synthetic insecticides is however associated with various negative impacts including adverse effects on humans, environment and biodiversity (Ton, 2000; Listorti and Doumani, 2001; Chopra *et al.*, 2005). Moreover, misuse of of synthetic insecticides has led to development of resistance that has been associated with control failures in SSA and Asia (Ekesi, 1999; Ton, 2000; Ulrichs *et al.*, 2001). Due to the aforementioned problems associated with excessive use of synthetic insecticides, there is an increased demand for alternative control strategies that are more sustainable and environment friendly.

Microbial control, including the use of entomopathogenic fungi (EPF), has been advanced as an environmentally acceptable alternative to synthetic insecticides in the management of insect pests (Wraight *et al.*, 2007; Zimmermann, 2007; Davidson, 2012; Vega *et al.*, 2012) Several entomopathogens have been reported to attack *M. vitrata* (Otieno *et al.*, 1983; Odindo *et al.*, 1989; Okeyo-Owuor *et al.*, 1991; Huang *et al.*, 2003). In earlier attempts to exploit these pathogens for the management of *M. vitrata*, Ekesi *et al.* (2002a) reported ovicidal effects of Nigerian isolates of *M. anisopliae* and *B. bassiana* against Nigerian populations of *M. vitrata* causing 71 – 100% egg infection and 61 – 100% deferred mortality in larvae. Additional studies using the same isolates against East African populations of *M. vitrata* yielded less than 45% pathogenicity on eggs (S. Ekesi *et al.*, unpublished data.). Available evidence suggests that various populations and host races of *M. vitrata* exist. For example, variations in response of West Africa, Southeast Asia, and South Asia populations to synthetic sex pheromone lures of *M. vitrata* have been

reported (Downham *et al.*, 2004; Hassan, 2007). It is probable that the difference in population may be responsible for the lack of efficacy of Nigerian isolates of EPF to East African populations of *M. vitrata*. There is need therefore to identify potent isolates of EPF that are efficacious against different populations and races of *M. vitrata*.

The ability of a candidate fungal isolate to infect and kill a target host pest, its amenability to mass production, and tolerance to environmental factors, are some of the important features to be considered in the selection of biocontrol agents (Inglis *et al.*, 2001; Wraight *et al.*, 2007; Zimmermann, 2007; Vega *et al.*, 2012). Entomopathogenic fungi are known to infect insects by means of the conidium germinating on the surface of insect cuticle and the germ-tube penetrating the cuticle into the haemocoel (Goettel and Inglis, 1997; Butt and Goettel, 2000; Wraight *et al.*, 2007; Vega *et al.*, 2012). This process is influenced by an array of factors including pathogen and insect host factors as well as environmental factors (Benz, 1987; Cory and Hoover, 2006; Wraight *et al.*, 2007; Jaronski, 2010; Vega *et al.*, 2012) which must be addressed in developing an EPF-based biopesticide.

1.2 Problem statement

Maruca vitrata is a pest of up to 39 host plants and is geographically distributed throughout the tropics and sub-tropics (Sharma, 1998; Sharma *et al.*, 1999). Although efforts have been made to improve the production of cowpea and other legumes, the pest remains a major constraint causing up to 80% yield losses in different parts of sub-Saharan Africa (SSA) and Asia (Karel, 1985; Afun *et al.*, 1991; Sharma, 1998; Sharma *et al.*, 1999). Regrettably, there is still lack of effective management methods and farmers continue to rely mainly on use of synthetic insecticides to control *M. vitrata* (Adati *et al.*, 2008; Ganapathy, 2010; Oyewale and Bamaiyi, 2013; Sabo, 2015), despite their negative impon on human health, non-target beneficials and the environmentally. Control failures associated with resistance development by *M. vitrata* to synthetic pesticides have also been reported (Okeyo-Owuor *et al.*, 1983; Atachi and Sourokou, 1989; Ekesi, 1999;

Ulrichs *et al.*, 2001). As farmers continue relying more on these products, their potential negative impact remain a great challenge. Although a potential advance in *M. vitrata* management has been achieved by engineering cowpea encoding genes that express the Cry1Ab delta endotoxin of *Bt* subsp. *kurstaki* (Popelka *et al.*, 2006; Chaudhury *et al.*, 2007; Adesoye *et al.*, 2008; Huesing *et al.*, 2011), this also has the risk of resistance development. Therefore, there is still a crucial need for alternative control measures that are more sustainable and environmentally acceptable. This study evaluates the use of EPF for management of *M. vitrata* on cowpea.

1.3 Research objectives

1.3.1 General objective

The overall objective of this study was to evaluate the use of entomopathogenic fungi for management of *M. vitrata* on cowpea.

1.3.2 Specific objectives

The specific objectives of this study were:

i) To identify potent isolates of entomopathogenic fungi that are pathogenic to *M. vitrata*

ii) To determine the effect of temperature on germination, radial growth and pathogenicity of candidate entomopathogenic fungi to *M. vitrata*

iii) To assess the role of host plant species on the pathogenicity of selected isolates of entomopathogenic fungi to *M. vitrata*

iv) To evaluate the performance of the selected entomopathogenic fungi in the management of *M. vitrata* on cowpea in the field.

1.4 Research Questions

The study was undertaken to answer the following questions:

i) Are there isolates of EPF that are pathogenic against *M. vitrata*?

ii) Does temperature affect germination, growth and pathogenicity of EPF against *M. vitrata;*

iii) Does host plant influence the germination, persistence and pathogenicity of EPF against *M. vitrata*

iv) Is the selected EPF isolate effective in the management of *M. vitrata* on cowpea in the field?

1.4 Justification of the study

Earlier attempts by Ekesi *et al.* (2002a) to exploit EPF to manage *M. vitrata* revealed that Nigerian isolates of *M. anisopliae* and *B. bassiana* were highly virulent against Nigerian populations of *M. vitrata,* causing 71 – 100% egg infection and 61 – 100% deferred larval mortality. These isolates however were found ineffective against the eggs of the East African populations of *M. vitrata* causing less than 45% eggs mortality (Ekesi *et al.*, unpublished data). There is evidence that various populations and host races of *M. vitrata* exist that may respond differently to various control strategies (Huang *et al.*, 2003; Margam *et al.*, 2011; Schläger *et al.*, 2012; Srinivasan *et al.*, 2012). Therefore, identification of potent isolates of EPF that are efficacious against different populations of *M. vitrata* is a critical step towards a successful IPM program for this pest. Fungal virulence, speed of kill and, amenability to mass production are some of the important parameters required for selecting candidate isolates for development of a biopesticide (Butt and Goettel, 2000). Environmental factors such as temperature, solar radiation, relative humidity, rainfall, and abiotic factors, are known to interact with pathogen and insect host factors to influence the efficacy of EPF (Butt and Goettel, 2000; Inglis *et al.*,

2001; Vega *et al.*, 2012). The present study was conceptualized to identify potent EPF against the Kenyan populations of *M. vitrata* also with the potential for wide-scale application in East and Southern Africa. It was also necessary to study the interaction of the selected fungal isolates with environmental factors namely temperature and host plants since these parameters influence efficacy of EPF in the field (Cory and Hoover, 2006; Wraight *et al.*, 2007; Cory and Ericsson, 2010; Jaronski, 2010; Vega *et al.*, 2012). Field testing was also considered as critical to further validate the efficacy of the candidate biopesticide in the past's natural habitat. Findings from this study should contribute towards the development of an environmentally sound and sustainable IPM package for *M. vitrata*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Importance of cowpea

Cowpea is an important multipurpose legume crop especially in Africa where it is consumed as food, serves as a source of income, fodder and is a rich source of soil nitrogen (Timko and Singh, 2008; Dugje *et al.*, 2009). It is a major staple food crop in SSA, especially in the dry savanna regions of West Africa. During the year 2012, Africa produced an estimated 5.4 million tonnes of dry cowpea constituting 94.5% of the world's production (5.7 million tonnes) (FAOSTAT, 2014). Nigeria was Africa's and the world's leading producer with a production of 2.5 million tonnes (43.8% of world's total production), harvested from 3.2 million hectares. Kenya ranked sixth in Africa and seventh globally, with a production volume of 113,961 tonnes from 215,269 hectares.

The seed, or grain as it is sometimes referred to, is the most important part of the cowpea plant for human consumption (Singh, 2005; Timko and Singh, 2008). In many parts of Africa and Asia, in addition to the seeds, the fresh or dried leaves are also consumed as a side dish or as part of a stew and provide significant nutritional value. The crop provides a rich source of plant proteins and vitamins for human, animal feed, and also serves as a major source of income (Singh, 2005; Adati *et al.*, 2008; Timko and Singh, 2008; Dugje *et al.*, 2009). Cowpea also plays an important role in providing soil nitrogen especially in areas where poor soil fertility is a problem. The crop has the ability to thrive in hot and dry conditions (Singh, 2005), and as such could significantly contribute to mitigation of climate change effects in SSA.

2.2 Constraints to cowpea production

A number of abiotic factors such as erratic weather conditions, low soil fertility, and high salinity, and biotic constraints such as diseases, insect pests, and parasitic plants impede cowpea production (Singh, 2005; Dugje *et al.*, 2009; Belmain *et al.*, 2013). Insect pests are considered the major constraint to cowpea production (Belmain *et al.*, 2013). Among the pests, the legume pod borer, *Maruca vitrata* is regarded as one of the most important (Adati *et al.*, 2008; Sureja *et al.*, 2010; Srinivasan *et al.*, 2012). Damage is caused by the larvae which feed on floral buds, flowers, and pods (Figure 2.1), and a single larva consumes 4 – 6 flowers before larval development is completed (Sharma, 1998). The flower bud stage is the most preferred plant part for oviposition by the female moth, and it is at this stage that the larvae cause substantial damage (Sharma, 1998; Sharma *et al.*, 1999). Larvae hatch from eggs in the early evening and wander on plant surfaces feeding on tender plant stems, terminal shoots, and peduncles during vegetative growth, and on flowers as they mature throughout the night (Sharma *et al.*, 1999; Ganapathy, 2010). This typical feeding habit protecs the larvae from natural enemies and other control agents, including insecticides (Usua and Singh, 1979). Damage to the flower buds causes flower shedding due to the destruction of the young flower parts enclosed in the sepals including the style, stigma, anther filaments, and ovary; limited feeding is also reported to occur on the internal components of the corolla (Sharma, 1998; Sharma *et al.*, 1999).

Varying losses in grain yield due to *M. vitrata* have been reported across different regions, but generally in the range of 20 – 80% (Sharma, 1998; Sharma *et al.*, 1999; Ganapathy, 2010). In Kenya, yield losses of up to 80% are common on different varieties of cowpea (Okeyo-Owuor and Ochieng, 1981). Ogunwolu (1990) reported seasonal variation in yield losses in Nigeria, where cowpea yield loss was 72% and 48% in 1985 and in 1986, respectively. He also established a provisional action threshold of 40% larval infestation in flowers.

Figure 2.1 Damage caused by *M. vitrata* on cowpea: (A) larva boring into flower bud; (B) larvae boring into mature flower; (C) abscission of young pods; (D) mature pod bored by feeding larvae. Photos: V. Tumuhaise.

2.3 The biology of *Maruca vitrata*

The biology of *M. vitrata* on cowpea and other legume crops has been extensively studied and/or reviewed by various authors (Okeyo-Owuor and Ochieng, 1981; Sharma, 1998; Naveen *et al.*, 2009; Ganapathy, 2010; Sureja *et al.*, 2010). *Maruca vitrata* undergoes a holometabolous development with its life cycle constituting the egg, larva, pupa, and adult

stages (Figure 2.2). Development from egg to adult takes 18 – 35 days, depending on host plant and temperature (Sharma, 1998; Naveen *et al.*, 2009; Ganapathy, 2010; Sureja *et al.*, 2010). Temperatures in the range of 22°C – 28°C are reported as optimal, while those above 34°C were lethal to the larvae (Jackai and Inang, 1992). The lower threshold temperature for development for pupa was 15.6°C – 17.8ºC, and the upper threshold ranged from 28°C – 34°C (Jackai and Inang, 1992).

The eggs of *M. vitrata* are light yellow, translucent, and have faint reticulate sculpturing on the delicate chorion, and measure 0.65 x 0.45 mm, laid either singly or in batches of 4 – 6 (Sharma, 1998; Shanower *et al.*, 1999; Naveen *et al.*, 2009; Ganapathy, 2010; Sureja *et al.*, 2010; Ranga Rao and Rameshwar Rao, 2013). However, although a batch of 16 eggs has been reported in some groups on terminal shoots, flower buds and pods (Shanower *et al.*, 1999). In their review, Shanower *et al.* (1999) noted that fecundity of more than 400 eggs per female had been reported earlier from laboratory studies. On the other hand, Naveen *et al.* (2009) reported a lower fecundity of 90 – 201 eggs per female when maintained on cowpea flowers in the laboratory. The eggs hatch in a period of 3 – 6.5 days (Sharma, 1998; Ganapathy, 2010; Sureja *et al.*, 2010).

Larvae of *M. vitrata* go through five instars lasting 8 – 14 days (Sharma, 1998; Ganapathy, 2010). Early instars are dull white, but later instars are black-headed, with irregularly shaped brown or black spots on the dorsal, lateral, and ventral surfaces of each body segment. When fully fed, the larvae can reach 17 – 20 mm long at the fifth instar stage (Sharma, 1998; Ganapathy, 2010). The early larvae prefer to feed on flower buds, flowers and tender pods but also feed on young tender shoots, peduncles, and stems (Usua and Singh, 1979; Sharma, 1998; Ganapathy, 2010). Because attack by the early instars on flower buds and flowers is internal, there is usually very little sign of damage until the flower wilts and drops (Abate and Ampofo, 1996). The larvae prefer concealment when feeding, and frequently web together flowers, pods, and leaves (Jackai and Daoust, 1986;

Sharma, 1998). This concealed feeding complicates control as pesticides and natural enemies cannot access the larvae easily (Jackai and Daoust, 1986).

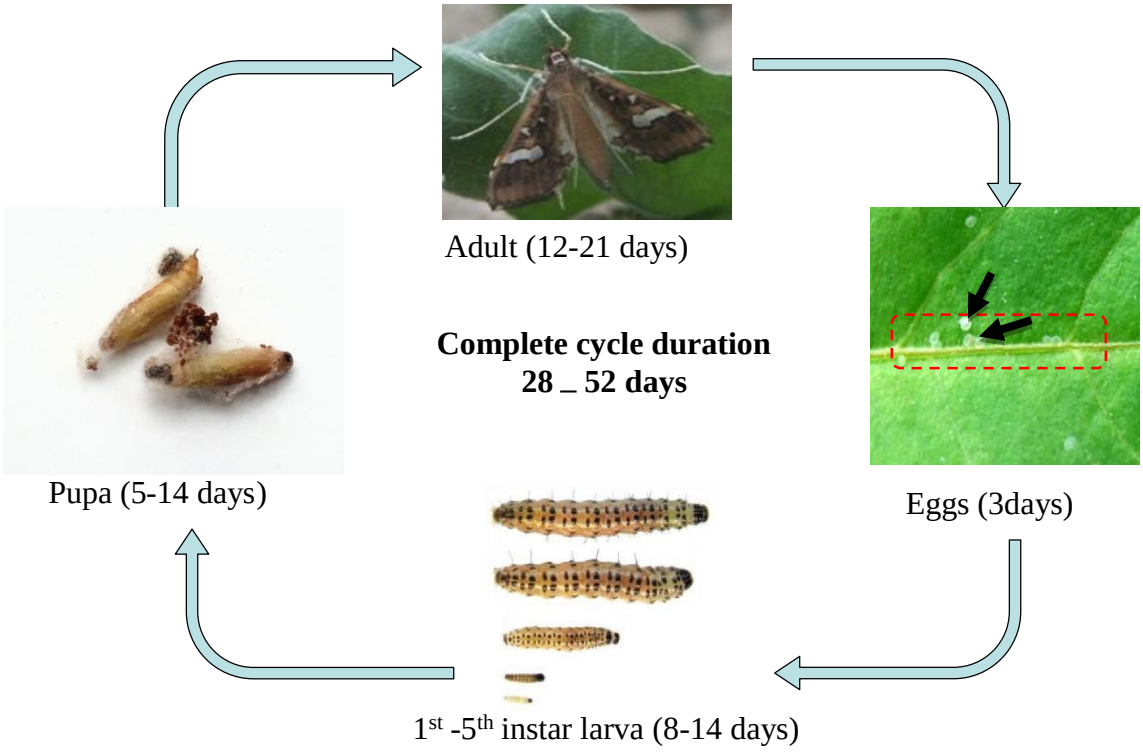

Figure 2.2 The generalized life cycle of *Maruca vitrata* (Sharma, 1998; Naveen *et al.*, 2009; Ganapathy, 2010; Sureja *et al.*, 2010). Black arrows on the green leaf point to the eggs. Photos: V. Tumuhaise (pupae, adult, eggs); Goergen, IITA (larvae).

When fully developed, the fifth instar larva becomes geostatic and subsequently enters a quiescent pre-pupal stage (Okeyo-Owuor and Ochieng, 1981). The pre-pupa is light-green in colour but changes after 24 h to light brown as it develops into a pupa (Okeyo-Owuor and Ochieng, 1981). Pupation occurs within a cocoon amongst webbed leaves, pods, under

leaf debris or soil (Jackai, 1981; Sharma, 1998; Ganapathy, 2010). The pre-pupal period lasts for two days while the pupal period lasts from 5 – 14 days (Okeyo-Owuor and Ochieng, 1981; Sharma, 1998). As the pupa develops, it progressively changes colour, weight and length with the weight and length reducing until it stays constant after 4 – 6 days. Colour changes from light brown through brick brown to dark brown mottled with black and yellow just before adult emergence (Okeyo-Owuor and Ochieng, 1981).

The adult moth has light brown forewings with white markings. The hind wings are pearly white with brown markings at the lateral edge. It rests with wings outspread and has a wingspan of 15 – 28 mm. Although the moth is chiefly nocturnal, it may also be seen during daytime. When reared on cowpea, male and female longevity were reported to vary from 6.1 – 10 .8 and 8.5 – 12.4 days, respectively. The entire life cycle of *Maruca vitrata* takes 28-52 days (Sharma, 1998; Shanower *et al.*, 1999; Naveen *et al.*, 2009; Ganapathy, 2010; Sureja *et al.*, 2010) (Figure 2.2).

2.4 Geographical distribution of *Maruca vitrata*

Maruca vitrata is a serious pest of several grain legumes in Asia, Africa, Australia, and the Americas with its recorded distribution stretching from the Cape Verde Islands in West Africa to Fiji and Samoa in the Far East, including the West Indies and Americas (Sharma, 1998; Sharma *et al.*, 1999) (Figure 2.3).

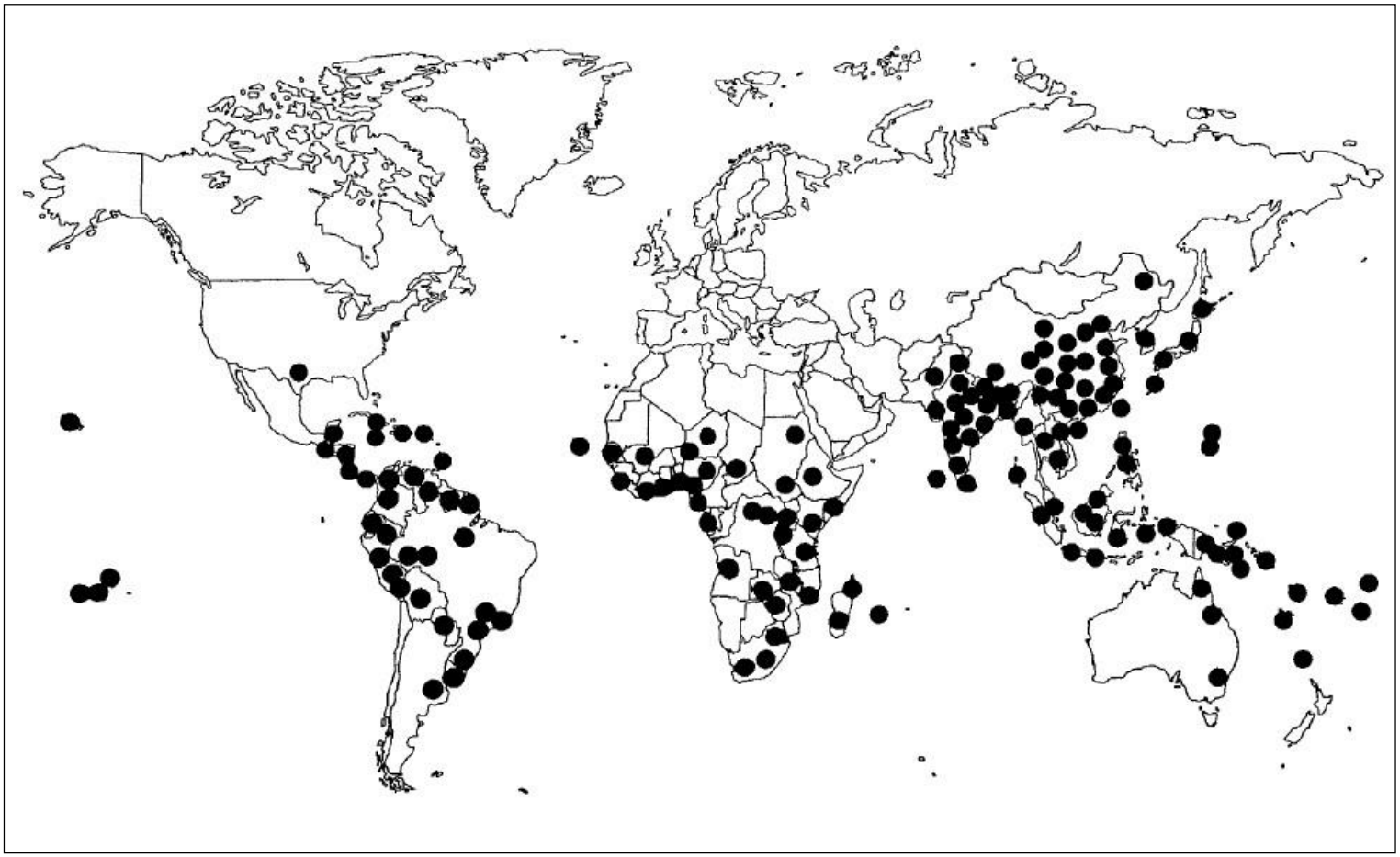

Figure 2.3 Geographical distribution of the legume pod borer, *Maruca vitrata* (Source: International Institute of Entomology, London, UK, 1996). Source: Sharma *et al.* (1999).

2.5 Host range of *Maruca vitrata*

The pest has been observed to feed on at least 39 host plants of which 37 are leguminous (Rathore and Lal, 1998; Sharma, 1998; Ranga Rao and Rameshwar Rao, 2013; Srinivasan, 2014), with *Cajanus cajan* (L.) Millsp., *Vigna unguiculata* Walp., *Phaseolus lunatus* (L.), and *Pueraria phaseoloids* (Roxb.) Benth, being the most frequent (Table 2.1). In the absence of cultivated host plants, the pest survives on alternative host plants like wild leguminous shrubs and trees(Tamò *et al.*, 2002; Ranga Rao and Rameshwar Rao, 2013; Srinivasan, 2014).The seasonal flowering pattern of the different host plants on a South-North gradient has been found to influence the migration of *M. vitrata* from the coast to the dry Savannas of West Africa (Bottenberg *et al.*, 1997). Thus the wide host range plays an important role in the population dynamics of this pest.

Table 2.1 Host range of the legume pod borer, *Maruca vitrata*

Common name	Scientific name	Reference
Papilionaceae		
Cowpea	*Vigna unguiculata*	Phelps and Oustihuizen (1958); Taylor (1967)
Green gram	*Vigna aureus*	Visvanathan *et al.* (1983)
Black gram	*Vigna mungo*	Taylor (1978); Das and Islam (1985)
Mung bean	*Vigna radiate*	Venkaria and Vyas (1985); Das and Islam (1985)
	Vigna triloba	Taylor (1967)
Pigeon pea	*Cajanus cajan* *Cajanus indicus*	Taylor (1967); Patel and Singh (1977) Taylor (1978)
Hyacinth bean	*Dolichos lablab*	Ramasubramanian and Sundara Babu (1988)
Country bean	*Lablab purpureus*	Das and Islam (1985)
Kidney bean	*Phaseolus vulagaris*	Rejesus (1978); Taylor (1978);

Common name	Scientific name	Reference
Lima bean	*Phaseolus lunatus*	Leonard and Mills (1931); Atachi and Djihou (1994)
Adzuki bean	*Phaseolus angularis*	Katayama and Suzuki (1984)
Broad bean	*Vicia foba*	Siddig (1982)
Yard long bean	*Vigna sinensis*	Satsijati *et al.* (1986)
Fusi-sasage	*Vigna vexillata*	Oghiakhe *et al* (1993)
Long bean	*Vigna sesquipedalis*	Ibrahim (1980)
Winged bean	*Psophocarpus tetragonolobus*	Taylor (1978)
Soya bean	*Glycine max*	Das and Islam (1985)
Ground nut	*Arachis hypogea*	Taylor (1978); Traore (1993)
African yam bean	*Sphenostylis stenocarpa*	Taylor (1978)
	Gliricidia sepium	Taylor (1978)
Grass pea	*Lathyrus sativus*	Das and Islam (1985)
Field pea	*Pisum sativum*	Das and Islam (1985)
	Pueraria phaseoloids	Atachi and Djihou (1994)
	Stizolobium sp.	Taylor (1978)
Valvet bean	*Mucuna sp.*	Taylor (1978)
	Tephrosia candida	Taylor (1978)
	Tephrosia purpurea	Taylor (1978)
	Crotalaria juncea	Jackai and Singh (1983)
	Crotalaria mucronata	Jackai and Singh (1983)
	Crotalaria incana	Jackai and Singh (1983)
	Crotalaria retusa	Atachi and Djihou (1994)
	Crotalaria amazonas	Jackai and Singh (1983)
	Crotalaria saltiana	Jackai and Singh (1983)
	Crotalaria misereniensis	Jackai and Singh (1983)
Cesalpinaceae	*Pansiana sp*	Taylor (1978)
Pedaliaceae	*Sesamum sp*	Taylor (1978)
Malvaceae	*Hibiscus sp*	Taylor (1978)
Mimosacea	*Escelerona dolabriformis*	Taylor (1978)

Source: Sharma (1998)

2.6 Management of *Maruca vitrata*

Management strategies for *M. vitrata* have always targeted the larval stage which causes damage to the crop. These larvae are only accessible during the very short exposure period on leaves, soon after hatching and before boring inside flowers or pods to start feeding (Jackai and Daoust, 1986; Sharma, 1998; Ganapathy, 2010). The concealed feeding behavior and webbing by the *M. vitrata* larvae complicates control of the pest as pesticides and natural enemies cannot easily penetrate to access the larvae (Jackai and Daoust, 1986).

2.6.1 Chemical control of *Maruca vitrata*

Synthetic pesticides are generally reported to be the major option used by farmers in an effort to control *M. vitrata*. For example, in Nigeria, Agwu (1997) found that 84.6% of the 130 farmers that participated in the survey used insecticides to control pests on cowpea. The author found this not surprising as yields of improved varieties were generally near zero without use of insecticides. It is documented that synthetic pesticides offer effective control against *M. vitrata* and other pests. For example, in the Philippines, Ulrichs *et al.* (2001) noted that plots protected with synthetic pesticides Methomyl® and Carbaryl® yielded more yardlong beans compared to those protected with the biological control agents *Bacillus thuringiensis* (Berliner) and *Trichogramma avanescens* (Westwood). The biocontrol agents however performed better than the untreated control. In another study, Rao *et al.* (2007) reported that chemical insecticides such as Spinosad® and Indoxacarb® can reduce *M. vitrata* populations by more than 70% on pigeon pea within two days after application.

In Africa, most of the smallholder who rely solely on pesticides for management of *M. vitrata* have little knowledge and guidance on the use of synthetic pesticides and apply insecticides haphazardly. Indiscriminate use of synthetic insecticides impacts negatively on human health and the environment including non-target beneficial organisms (Ton,

2000; Chopra *et al.*, 2005). For example, in Benin more than 87 persons were reported to have died in 1999 due to pesticide-contaminated vegetables grown in cotton/cowpea fields (Ton, 2000). Cases of control failures associated with the development of resistance due to misuse of pesticides have also been reported (Ekesi, 1999; Ulrichs *et al.*, 2001). Thus, there is an increased demand for alternative control strategies that are more sustainable and environment friendly.

2.6.2 Cultural control of *Maruca vitrata*

Planting time, intercropping, and weeding are some of the agronomic practices considered important in the management of *M. vitrata* (Alghali, 1992; Ekesi *et al.*, 1996; Karungi *et al.*, 2000; Hassan, 2009; Oso and Falade, 2010). For example, cowpea monocrops are reported to be more affected by *M. vitrata* than intercropped cropping systems (Alghali, 1992). Late planted cowpea is reported to suffer more infestation by *M. vitrata* as compared to the early planted crop, and this is attributed to the fact that *M. vitrata* populations tend to build up as the season advances (Ekesi *et al.*, 1996).

The role of intercropping in the management of *M. vitrata* remains inconclusive. For example, Oso and Falade (2010) studied the effects of variety and spatial arrangement on pest incidence, damage and subsequent yield of cowpea in a cowpea/maize intercrop in Nigeria, and found no significant differences in the population density of *M. vitrata* among the 1:1 and 2:3 maize/cowpea intercrops. However, a significantly higher population of *M. vitrata* was observed on *cv*-Ife brown than Tvu-13076 varieties leading to the conclusion that intercropping may not necessarily reduce pest load in any situation. From yet another study still in Nigeria, Hassan (2009) reported a significantly lower population of *M. vitrata* in cowpea-sorghum intercrop than sole cowpea crop in 2007, and this was the reverse of what was recorded during the 2006 cropping season. Thus cultural control methods remain unpredictable and so may not singly be relied on for the management of *M. vitrata*.

2.6.3 Use of resistant cowpea varieties

Host plant resistance has always been viewed as the most reliable strategy in pest management. However, there are no varieties of cowpea reported to have sufficient resistance levels against *M. vitrata*. Cowpea lines TVu-946, TVu-13271, and VITA-5 bred by IITA are only reported to express low levels of resistance against *M. vitrata* (Singh, 2005). A potential advance in *M. vitrata* management has been achieved by engineering cowpea encoding genes that express the Cry1Ab delta endotoxin of *B. thuringiensis* subsp. *kurstaki* (Popelka *et al.*, 2006; Chaudhury *et al.*, 2007; Adesoye *et al.*, 2008; Huesing *et al.*, 2011). This however has the potential risk of resistance development.

2.6.4 Use of botanical insecticides

Various studies have demonstrated the potential of plant extracts and volatiles against *M. vitrata* (Ekesi, 2000; Oparaeke, 2006; Srinivasan, 2012; Srinivasan *et al.*, 2012). Neem-based biorationals are among the most studied in the management of insect pests, including *M. vitrata*. For example, in a study to evaluate the effect of crude aqueous extracts of some tropical plants for their insecticidal properties, Oparaeke (2006) showed that extracts of neem, *Azadirachta indica* (A. Juss), bread fruit, *Artocarpus altilis* (Park) and fermented cassava tuber, *Manihot esculentus* (Crantz) significantly reduced both *M. vitrata* and *Clavigralla tomentosicollis* (Stål.) on cowpea plants. Pod damage was also considerably reduced (< 35%) with consequent increases in grain yields and seed quality in plots treated with seed extracts of neem and breadfruit.

2.6.5 Biological control of *Maruca vitrata*

Several parasitoid, predator and pathogen species have been recorded on *M. vitrata* (Odindo *et al.*, 1989; Okeyo-Owuor *et al.*, 1991; Huang *et al.*, 2003). Apparently, all life stages of *M. vitrata* are susceptible to parasitism (Tamò *et al.*, 1997; Sharma, 1998). In

Kenya, seven parasitoids, two predators, one nematode, and several pathogens were recorded (Otieno *et al.*, 1983; Odindo *et al.*, 1989; Okeyo-Owuor *et al.*, 1991). The pupal endoparasitoid, *Antrocephalus* sp. was the predominant natural enemy, while *Nosema* sp. and *Bacillus* sp. caused the highest natural mortality (Sharma, 1998). The dominant species recorded in Benin was *Phanerotoma leucobasis* Kriechbaumer (Hymenoptera: Braconidae) parasitizing on average 5.6% of *M. vitrata* larvae in cowpea fields (Tamò *et al.*, 2002; Arodokoun *et al.*, 2006). However, overall parasitism rates under field conditions are generally low, ranging between < 4 and 15% (Okeyo-Owuor *et al.*, 1991; Arodokoun *et al.*, 2006; Adati *et al.*, 2008; Dannon, 2011). Most recently, three species-specific parasitoids (*A. taragamae, T. javanus* and *T. marucae*) of *M. vitrata* have been identified (Srinivasan *et al.*, 2012) and are under evaluation for use in Africa. However, parasitioids alone cannot be used as a sole management method but must be harnessed within the context of effective IPM package for *M. vitrata.*

2.6.6 Entomopathogens

Microbial control agents, including bacteria, fungi, nematodes, protozoa and viruses, are among natural enemies of *M. vitrata* (Otieno *et al.*, 1983; Odindo *et al.*, 1989; Okeyo-Owuor *et al.*, 1991; Sharma, 1998; Huang *et al.*, 2003), and are more environmentally acceptable and sustainable alternatives to chemical insecticides (Wraight *et al.*, 2007; Cory and Ericsson, 2010; Jaronski, 2010; Shapiro-Ilan *et al.*, 2012). Among these microorganisms, fungi constitute the largest single group of insect pathogens with over 700 species of fungi known to infect insects (Wraight *et al.*, 2007). There are a wide range of commercial microbial-based biopesticides registered for insect pest control. For example, the bacterium, *Bt* subsp. *kurstaki* is registered in Kenya, under various trade names, for example Halt and Thuricide, targeting different pests of agricultural importance including African bollworm, *Helicoverpa armigera*, *Spodoptera exigua,* diamond back moth and other Lepidopteran larvae. Fungal-based biopesticides are also registered with *B. bassiana*-based products targeting aphids, the diamond back moth, and sucking insect

pests, while *Paecilomyces lilacinus* products target nematodes (Table 2.2). More recently, a private company in Kenya (Real IPM (Kenya) Ltd., in collaboration with *icipe*, has undertaken commercial production of three biopesticides based on *M. anisopliae* isolates ICIPE 69, ICIPE 62, and ICIPE 78, respectively (Table 2.3).

Table 2.2 Selected microbial pesticides registered in Kenya by the year 2010

Active ingredient	Taxon	Trade name	Target pest
Bacillus thuringiensis subsp. *aizawai*	Bacterium	Florbac Xentari	Coffee giant looper
Bacillus thuringiensis subsp. *israelensis*	Bacterium	Bacticide	Mosquito larvacide
Bacillus thuringiensis subsp. *kurstaki*	Bacterium	Biolep DiPel Halt Thuricide	Thrips, African bollworm, *Helicoverpa armigera*, *Spodoptera exigua*, Lepidoptera larvae, diamond black moth
Bacillus thuringiensis subsp. *Kurstaki* SA11	Bacterium	Delfin	Diamond back moth, coffee giant looper
Beauveria bassiana GHA	Fungus	Bio-power Botanigard	Aphid, Diamond back moth, sucking insect pests
Paecilomyces lilacinus	Fungus	Bio-Nematon	Root knot nematode

Modified from Gwynn and Maniania (2010)

Table 2.3 Mycopesticides produced by Real IPM (Kenya) Ltd in collaboration with *icipe* – Kenya

Fungus (active ingredient)	**Trade name**	**Target pest**
Metarhizium anisopliae isolate ICIPE 69	• Real *Metarhizium anisopliae* 69 • Campaign	• Kills thrips, weevils, whiteflies and mealy bugs. • It is compatible with beneficial insects, including predatory mites and bees.
Metarhizium anisopliae isolate ICIPE 62	Real *Metarhizium anisopliae* 62	• Aphids. • It is compatible with aphid predators and parasitic wasps
Metarhizium anisopliae isolate ICIPE 78	Achieve (Metarhizium 78)	• Kills spider mite (*Tetranychus urticae*) and also *T. evansii*. It also controls varroa mites in bee hives. • It is compatible with predatory mites (*Phytoseiulus* and *Amblyseius* spp.) and bees.

Source: (www.realipm.com)

2.6.6.1 Entomopathogenic bacteria

Bacillus thuringiensis (*Bt*), which occurs naturally in the soil around the world, is among the most extensively used entomopathogens for control of insect pests in crops, forests, and the aquatic environments (Lacey and Siegel, 2000; Hilbeck and Schmidt, 2006; Srinivasan, 2012). Pest insects in the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, and Mallophaga are indicated to be susceptible to strains of *Bt* (Martin and Traverst, 1989; Stenersen, 2004; Schünemann *et al.*, 2014). Nematoda and

Protozoa are also reported to be susceptible to some *Bt* strains (Stenersen, 2004). When target insects ingest either the bacterium or the protein produced by the bacterium, it results in cessation of feeding and paralysis of the gut that retards the passage of food and allows the spores to germinate (Stenersen, 2004; Schünemann *et al.*, 2014).

Studies have demonstrated that *M. vitrata* is susceptible to endotoxins produced by *Bt* (Adesoye *et al.*, 2008; Srinivasan, 2008, 2012). For example, Srinivasan (2008) assessed the susceptibility of *M. vitrata* to *Bt* δ-endotoxins and found that δ-endotoxins Cry1Ab was the most potent toxin with LC_{50} of 0.207 ppm. Synergistic interaction have been reported between *Bt*-based biopesticides and other biopesticides or biocontrol agents (Wraight and Ramos, 2005; Srinivasan, 2012). This compatibility demonstrates a great potential for development of an effective biopesticide for a sustainable IPM system. Several commercial products of *Bt* are registered for use against insect pests worldwide (Stenersen, 2004; Schünemann *et al.*, 2014), including in Kenya (Gwynn and Maniania, 2010).

2.6.6.2 Entomopathogenic viruses

Viruses that contain ribonucleic acid (RNA) as their genetic material and thus grouped as RNA viruses comprise a wide variety of infectious agents, many of which induce disease in plant, vertebrate, and invertebrate hosts (Chen *et al.*, 2012). The RNA viruses infecting insect pests are currently limited to six families, namely *Nodaviridae* (genus *Alphanodavirus*), *Dicistroviridae* (genera *Cripavirus* and *Aparavirus*), *Flaviviridae* (genus *Flavivirus*), *Iflaviridae* (genus *Iflavirus*), *Tetraviridae* (genera *Betatetravirus* and *Omegatetravirus*), and *Reoviridae* (genus *Cypovirus*) (Chen *et al.*, 2012).

RNA viruses have been found effective against *M. vitrata* (Tamò *et al.*, 2003; Lee *et al.*, 2007; Adati *et al.*, 2008; Srinivasan *et al.*, 2012). For example, Tamò *et al.* (2003) reported a cypovirus (CPV) that was found in southern Benin in 1998 infecting larvae of *M. vitrata*

on wild leguminous plants. However, this group of entomopathogenic RNA viruses has received limited attention in biological control programmes because of their infection within an insect population is generally chronic rather than epidemic, and less lethal (Adati *et al.*, 2008).

More recently, a nucleopolyhedrovirus (*MaviMNPV*) was isolated from diseased larvae of *M. vitrata* in Taiwan, and later laboratory bioassays revealed that first instar *M. vitrata* larvae were the most susceptible stage to this NPV (Lee *et al.*, 2007). Formulations of MaviMNPV have been found to be effective against *M. vitrata*, either alone or in combination with other biopesticides (Srinivasan, 2012). Generally, the use of RNA viruses as microbial control agents presents unique challenges and concerns including the taxonomic similarity with human RNA viruses and the possible exchange of genetic material, a high genome mutation rate, and difficulties in producing large, stable quantities of virus that hamper their development and use in insect control (Chen *et al.*, 2012).

2.6.6.3 Entomopathogenic fungi

Among micro-organisms, fungi constitute the largest single group of insect pathogens with over 700 species of fungi known to infect insects (Wraight *et al.*, 2007). Entomopathogenic fungi are unique from other entomopathogens in that they infect their hosts primarily through the external cuticle, although a few taxa (e.g. *Culicinomyces*) are able to invade through the alimentary canal (Inglis *et al.*, 2001). *Beauveria* spp. and *Metarhizium* spp., belonging to order Hypocrealea, are perhaps the most studied fungi in the field of entomopathology. Insect pests infected with these fungi experience sublethal effects 1 – 4 days prior to death. They exhibit reduced feeding activity and fecundity, which reduces their pest status while they are still alive (Roy *et al.*, 2006).

Metarhizium anisopliae and *B. bassiana* are among the Hyphomycetes reported to have wide host range among invertebrates (Butt and Goettel, 2000; Wraight *et al.*, 2007),

although some isolates can be host specific. *Beauveria bassiana* and *M. anisopliae* have both been reported to have activity against *M. vitrata*, and as such, they have been proposed as an important arsenal for managing the pest. In earlier attempts to exploit these pathogens for the management of *M. vitrata*, (Ekesi *et al.*, 2002a) reported ovicidal effects of Nigerian isolates of *M. anisopliae* and *B. bassiana* against Nigerian populations of *M. vitrata* causing 71 – 100% egg infection and 61–100% deferred mortality in larvae. The isolates were also pathogenic to the pod sucking bug, *Clavigralla tomentosicollis*.

A number of *B. bassiana* and *M. anisopliae* isolates from the ICIPE Arthropod Germplasm have been found to be pathogenic to various insect pests such as *Cylas puncticollis* (Boheman) (Ondiaka *et al.*, 2008), *Liriomyza huidobrensis* (Blanchard) (Migiro *et al.*, 2010), *Tetranychus urticae* (Koch) (Maniania *et al.*, 2008); *Tetranychus evansi* (Baker and Pritchard) (Wekesa *et al.*, 2006; Maniania *et al.*, 2008), and *Megalurothrips sjostedti* (Trybom) (Ekesi *et al.*, 2000a). However, no information is available on the effectiveness of these fungal isolates against *M. vitrata*.

2.7 Mode of action of entomopathogenic fungi

The mode by which EPF infect their host insects is well described by several authors (e.g. Wraight *et al.* 2007; Zimmermann 2007; Sandhu *et al.* 2012; Shahid *et al.* 2012; Vega *et al.* 2012). Briefly, once the conidium lands on to the insect's cuticle, the infection process commences with the initial events of host recognition and attachment to the cuticle that is normally achieved through the secretion of mucilage. The conidium subsequently germinates to form a germ tube and appressorium. A penetrating hypha breaches the cuticular layers by secreting enzymes that hydrolyze the epidermis of the insect, the most important of which being lipases, proteases and chitinase. The gem tube then reaches the hemocoel where blastospores or hyphal bodies are formed *in vivo*. Fungal growth continues and moves throughout the hemocoel, invading organs, disrupting metabolic processes, and possibly producing toxic metabolites, eventually causing death (Figure 2.4)

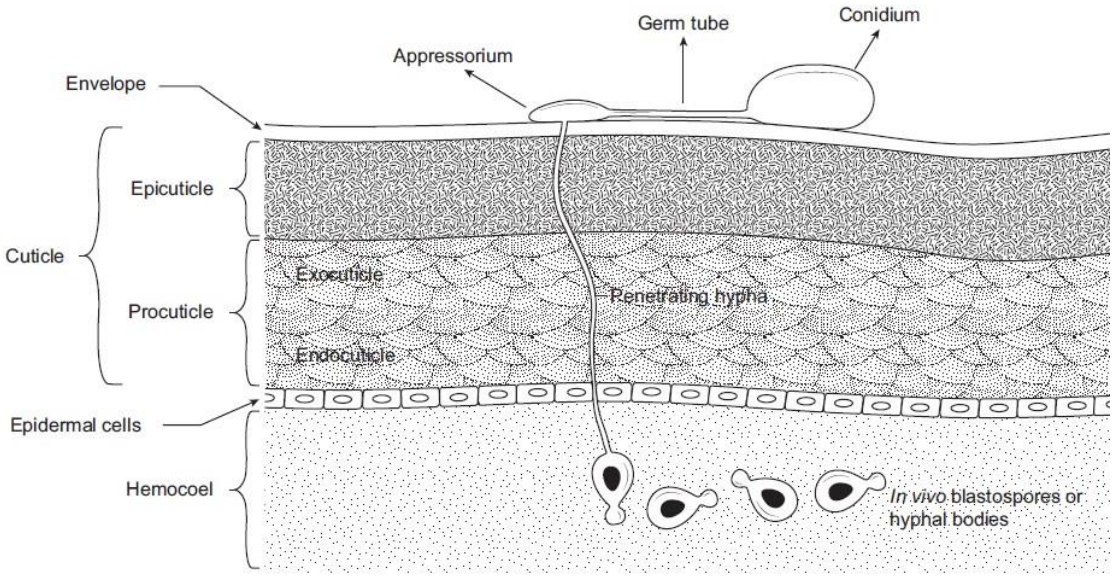

Figure 2.4 Generic model showing the infection process for fungal entomopathogens. Source: Vega *et al.* (2012)

2.8 Factors affecting efficacy of entomopathogenic fungi as biocontrol agents

The key factors that have been shown to affect the efficacy of entomopathogenic fungi include solar radiation, microbial antagonists, host behavior, physiological condition and age, pathogen vigor and age, leaf surface chemistry, phylloplane microbiota and presence of pesticides, temperature, humidity and inoculum thresholds (Cory and Ericsson, 2010; Jaronski, 2010; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012). The environmental factors come into action as soon as the spores are applied to foliage, through the initial infection process, to the point they are inside the body of the host insect (Jaronski, 2010). As such, most conidia are likely to disintegrate quickly in the environment, and only minimal proportions will presumably succeed in infecting new hosts (Meyling and Eilenberg, 2007). Whereas, the plant can directly influence pathogen infectivity and viability, entomopathogen influence on the plant usually occurs indirectly via its effect on the insect

(Figure 2.5). Plant phytochemicals can affect entomopathogens and their hosts negatively or positively: the outcome of the interaction, in terms of insect fitness, is dictated by which player is most strongly affected by the prevailing plant chemistry (Cory and Hoover, 2006). The tritrophic interaction can be further influenced by the action of other natural enemies and infection of the plant host by phytopathogens, which, in turn, are likely to affect insect behaviour and plant quality (Cory and Hoover, 2006).

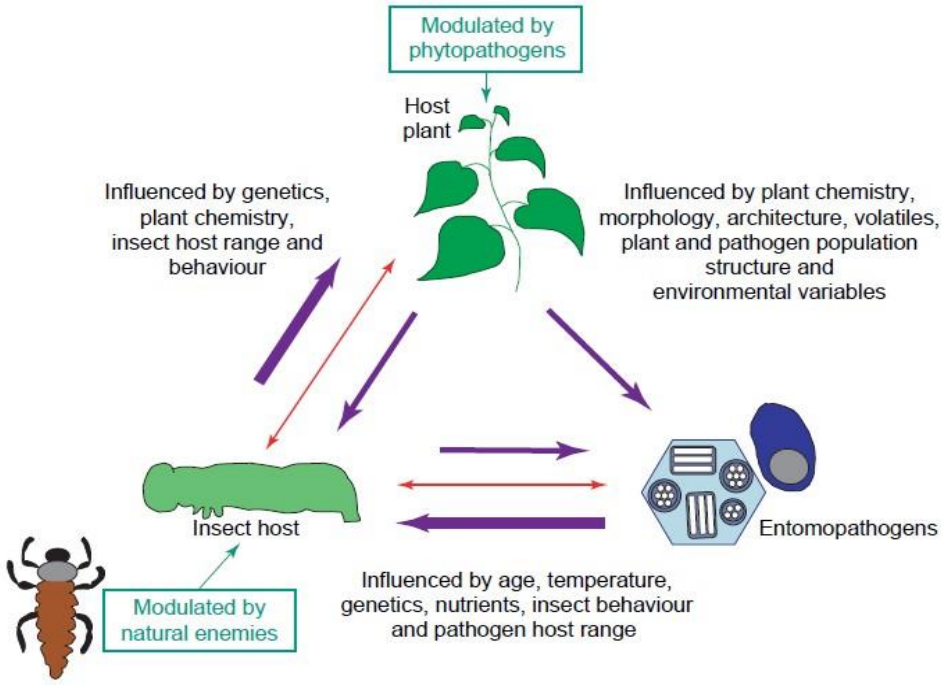

Figure 2.5 The theoretical framework in which insects, plants and entomopathogens can interact and the factors that influence the outcome. Arrow size represents the probable strength of the response and the double-headed arrows indicate where coevolution is expected. Source: Cory and Hoover (2006).

2.8.1 The pathogen

The ability of entomopathogens to produce epizootics among the host population is influenced by pathogen-related factors such as genetics, latency, virulence, pathogen density, dispersal, host range, persistence and the capacity to disperse within the host's habitat (Inglis *et al.*, 2001; Wraight *et al.*, 2007; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012). Susceptibility of most insects is dependent on spore dosage and persistence of the fungal propagules (Butt and Goettel, 2000; Inglis *et al.*, 2001). It is presumed that a threshold exists whereby a certain number of propagules are necessary to overcome the host (Butt and Goettel, 2000). Propagule densities must therefore be sufficiently high, especially in a field setting, to ensure a high probability that an insect will come in contact with an adequate number of propagules to exceed the inoculum threshold (Inglis *et al.*, 2001). The ability of an entomopathogenic fungal species to persist in an environment increases the probability of an insect coming in contact with sufficient propagules to cause disease (Inglis *et al.*, 2001).

Most entomopathogenic fungi gain entry to the hemocoel by penetrating the host cuticle using a combination of hydrolytic enzymes and mechanical force (Cory and Hoover, 2006; Wraight *et al.*, 2007; Shahid *et al.*, 2012; Vega *et al.*, 2012). Host death is due to a combination of toxin, physical obstruction of blood circulation, nutrient depletion and invasion of organs (Inglis *et al.*, 2001; Cory and Hoover, 2006; Wraight *et al.*, 2007; Shahid *et al.*, 2012; Vega *et al.*, 2012). Fungal entomopathogens produce many secondary metabolites although, for the most part, the role of these metabolites in pathogenesis remains unclear (Molnár *et al.*, 2010). *Beauveria* spp. have been reported to produce several different cyclodepsipeptides including beauvericin, beauverolides, bassianolides, and bassiatin (Vega *et al.*, 2012). Cyclodepsipeptides act as ionophore antibiotics owing to their selective interactions with potassium or sodium ions, thus altering the permeability of cell membranes (Ngoka *et al.*, 1999). Beauvericin is the most studied *B. bassiana*-produced cyclodepsipeptide, and some of the cyclodepsipeptides produced by *Beauveria*

spp. have also been found to be produced by other fungi such as *Lecanicillium* spp., *Cordyceps* spp., *Fusarium* spp., and *Isaria* spp. (Vega *et al.*, 2012). Destruxins, another group of cyclodepsipeptides produced by various fungi, were first isolated from *M. anisopliae* and some of the 38 different destruxins or destruxin analogues have been shown to be insecticidal (Vega *et al.*, 2012).

2.8.2 The insect host

Fungi, as a group, have one of the widest host ranges among the pathogens of arthropods although host spectra vary widely, depending on fungal species. *Beauveria bassiana* and *M. anisopliae* have much wider host ranges, spanning numerous orders within the Arthropoda (Inglis *et al.*, 2001). A complex array of physiological and morphological factors influence the susceptibility of insect pests to EPF (Jaronski, 2010; Inglis *et al.*, 2012; Shahid *et al.*, 2012; Ortiz-urquiza and Keyhani, 2013). Some examples include pest population growth characteristics, population composition, host population density and distribution, and host behavior (Fuxa, 1987; Wraight *et al.*, 2007; Shapiro-Ilan *et al.*, 2012). Other important host factors include age, nutrition, genetics and exposure to injuries caused by mechanical, chemical or non-microbial agents such as predators and parasites (Inglis *et al.*, 2001; Shapiro-Ilan *et al.*, 2012).

Insect nutrition is a very important factor regulating the susceptibility of insects to entomopathogens (Butt and Goettel, 2000; Inglis *et al.*, 2001; Wraight *et al.*, 2007; Shapiro-Ilan *et al.*, 2012). Inadequate nutrition often leads to increased susceptibility, as the utilization of resistant plant genotypes to induce nutritional stress can also substantially enhance the efficacy of entomopathogens (Butt and Goettel, 2000; Inglis *et al.*, 2001; Shahid *et al.*, 2012; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012). Conversely, diet can also decrease the susceptibility of insect pests to entomopathogenic fungi. For example, Ekesi *et al.* (2000a) found that flower thrips, *Megalurothrips sjostedti* were less susceptible to

M. anisopliae on certain cowpea cultivars because of plant-derived fungistatic compounds.

Age is another important factor influencing susceptibility of an insect to fungal infection, as the degree of susceptibility varies across the different stages in an insect's life cycle (Butt and Goettel, 2000; Inglis *et al.*, 2001). For instance, larvae of the thrips, *Frankliniella occidentalis* (Pergande) were found less susceptible to *V. lecanii* and *M. anisopliae* than adults, while later instars were less susceptible than earlier instars (Vestergaard *et al.*, 1995). On the other hand, (Feng *et al.*, 1985) reported that young larvae of the European corn-borer, *Ostrinia nubilalis* (Hubner) are more susceptible to *B. bassiana* than older larvae.

Increased host density favours infection through increased contact between uninfected and infected hosts, and between uninfected hosts and pathogen units (Fuxa, 1987; Inglis *et al.*, 2001, 2012). High host density can also stress insects in the population, predisposing them to disease. The many host individuals also provide substrate and nutrients more readily available for pathogen growth and reproduction, (Fuxa, 1987) thus increasing the quantity of inoculum available in the habitat to further cause infection.

Behavioural traits of some insects such as grooming in termites infected with *M. anisopliae*, and summit disease syndrome in grasshoppers infected with *Entomophaga grylli* (Fres.) promote epizootic development and can affect the dispersal of an entomopathogen (Inglis *et al.*, 2001). On the other hand, sanitation behaviour exhibited by many social insects can limit the spread of an entomopathogen (Inglis *et al.*, 2001; Vega *et al.*, 2012). For example, some insects such as flies and locusts once infected modify their behavior to elevate body temperatures, for example by basking in the sun, to a level that is adverse to the entomopathogenic fungus in the hemocoel (Roy *et al.*, 2006; Jaronski, 2010; Vega *et al.*, 2012; Ortiz-urquiza and Keyhani, 2013).

Physicochemical properties of the insect cuticle interact with EPF to influence the infection process (Figure 2.6). While epicuticular substances stimulate spore germination, some insects possess fungistatic compounds (Jaronski, 2010). For instance, cuticular extracts from larval *Helicoverpa zea* (Boddie) inhibited *B. bassiana* conidial germination (Smith and Grula, 1982), while conidial germination of *M. anisopliae* on *N. viridula* cuticle was much lower (only 5 – 20%) than on other insect cuticle substrates, parallel to reduced infectivity for that insect (Sosa-Gomez *et al.*, 1997). The latter was attributed to presence of the aldehyde, (E)-2-decenal. Nevertheless, current evidence does indicate that the insect cuticle surface can mediate successful infection, and the existence of highly pathogenic isolates for most insects further implies that fungi can be found for which these barriers are unimportant (Jaronski, 2010).

Once the fungal entomopathogen reaches the hemolymph, a range of immune responses can be initiated, of which some are general antimicrobial responses while some specifically target the invading fungus; for example, fungal units can be cellularly phagocytosed or encapsulated (Vega *et al.*, 2012). Interestingly, *M. anisopliae* and *B. bassiana* are capable of avoiding encapsulation in the hemocoel, and this adaptation has been hypothesized to be a consequence of these fungi being facultative entomopathogens in soil environments where they can survive encapsulation by soil amoeboid predators (Bidochka *et al.*, 2001).

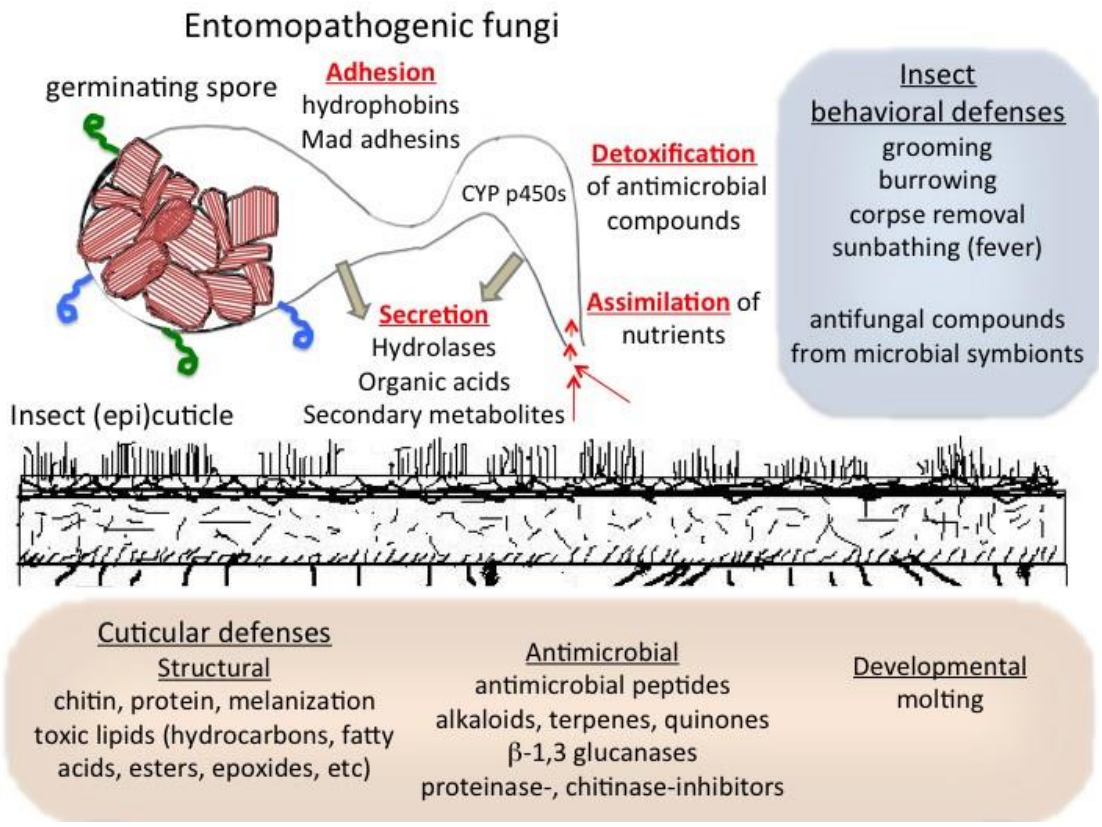

Figure 2.6 An overview of surface interactions between entomopathogenic fungi and the insect cuticle and host behaviors. Source: Ortiz-urquiza and Keyhani (2013)

2.8.3 The host plant

In a review of fungal entomopathogens in a tritrophic context, Cory and Ericsson (2010) noted that plant-mediated effects on fungal entomopathogens could either be direct or indirect. They summarized the potential direct effects as: (1) plant exudates affecting the conidia directly, (2) herbivore-induced plant volatiles affecting sporulation or germination, (3) leaf topology and surface chemistry, influencing the rate of spore acquisition by the host insect, (4) plant architecture altering spore persistence, and (5) leaf modifications of microclimate and thus spore germination. On the other hand, the authors highlighted potential indirect effects as: (1) plant quality, either allelochemicals or

nutrients, altering insect condition (e.g. immunity) and thus disease resistance, (2) nutritional quality altering insect morphology (e.g. cuticle depth) which would influence the infection process, (3) changes in insect growth rate, which might alter the exposure of the insect to fungal entomopathogens, and (4) plant structure altering insect behaviour, and thus fungal encounter rate (Cory and Ericsson, 2010).

There is documented evidence that demonstrates the effects of host plants on the efficacy of entomopathogenic fungi. For example, Klingen *et al.* (2002) assessed the effect of different brassicaceous plants on colony forming units (CFU) for *M. anisopliae*, and observed that pots with plant treatments had significantly more CFUs than the control. Additionally, significantly more *M. anisopliae* CFUs were found in pots containing *E. vesicaria sativa* than *B. vulgaris*. (Poprawski *et al.*, 2000) also found that third-instar nymphs of *Trialeurodes vaporariorum* reared on cucumber were highly susceptible to infection by both *B. bassiana* and *Paecilomyces fumosoroseus* onto cucumber while those reared on tomato plants were significantly less susceptible to infection. (Butt and Goettel, 2000) indicated that larvae growing on more favourable plant species could be better able to mount a successful defensive reaction to pathogens, or have a shorter inter-moult period through which the insect reduces the quantity of infective propagules on the exuviae.

Presence of other microorganisms such as bacteria, viruses, nematodes, and other fungi within the microhabitat of the target insect host may result into mixed infection. Differences in fungal virulence have been reported in situations of inter- and intraspecific mixed infection (Brinkman and Gardner, 2000; Thomas *et al.*, 2003; Jaronski, 2010; Staves and Knell, 2010). For example, Brinkman and Gardner (2000) while studying the enhanced activity of *B. bassiana* to red imported fire ant workers (*Solenopsis invicta* L.) infected with *Thelohania solenopsae* observed that ants from the microsporidian-infected colonies were 4.5 times more susceptible to *B. bassiana* than those from the healthy colonies.

Pesticides and fungicides are usually applied in conventional farming practices, and their presence on the phylloplane might negatively affect the populations of entomopathogenic fungi with reduced pest regulation potential as a consequence (Meyling and Eilenberg, 2007). Studies have been done to evaluate the effects of chemical insecticides on entomopathogenic fungi (Akbar *et al.* 2012; Niassy *et al.* 2012b). For instance, Akbar *et al.* (2012) observed that insecticides chlorpyrifos, match, profenofos, and metalaxyl + mancozeb were detrimental to germination and growth of isolates of *M. ansopliae*, while spinosad and indoxacarb were significantly compatible, with no deleterious effects on germination and growth.

2.8.4 The environment

The ability of an entomopathogen to tolerate environmental constraints is one of the key requirements for successful pest control (Cabanillas and Jones, 2009; Bouamama *et al.*, 2010). The major microclimatic constraints include temperature, sunlight, humidity and rainfall (Benz, 1987; Inglis *et al.*, 2001; Wraight *et al.*, 2007; Zimmermann, 2007; Jaronski, 2010; Vega *et al.*, 2012).

2.8.4.1 Temperature

Several studies have demonstrated that temperature influences such crucial events as spore germination, host penetration and growth in the host that determine the efficacy of entomopathogenic fungi (Benz, 1987; Ouedraogo *et al.*, 1997; Li and Feng, 2009; Rangel *et al.*, 2010; Vega *et al.*, 2012). Most entomopathogenic fungi have a wide range of temperature tolerances although deviations from generalized trends have been observed, across and within species (Fargues *et al.*, 1997; Vidal *et al.*, 1997; Wraight *et al.*, 2007). The authors also indicated that temperatures optimal for infection, growth, and sporulation are usually much more restricted, generally in the range of 20 – 30^{o}C. For example, in one

study involving 65 isolates of *B. bassiana*, Fargues *et al.* (1997) observed that 50 isolates had an upper threshold of 35 – 37ºC; 12 had a threshold of 32 – 35ºC; while one had a threshold of 30 – 32ºC. In another study by Ouedraogo *et al.* (1997) involving 36 isolates of *M. anisopliae* and *M. flavoviride*, majority grew between 11 and 32ºC while several other isolates grew in temperature ranges of 8 – 37ºC. These and several other studies demonstrate that temperature is a variable for which generalizations are difficult to make (Vega *et al.*, 2012). Therefore, in the process of evaluating the ability of candidate fungus as a potential biopesticide, it is important to consider not only its virulence to the target insect but also suitability for environmental conditions occurring in the insect habitat (Cabanillas and Jones, 2009). Temperature affects pathogen as well as insect host processes, (Inglis *et al.*, 2001; Wraight *et al.*, 2007; Jaronski, 2010) which together interact to determine the degree of susceptibility and disease development. High temperatures accelerate insect development, and will reduce the time between moults, which can subsequently reduce the prevalence of infection due to loss of inocula on exuviae (Butt and Goettel, 2000; Inglis *et al.*, 2012; Ortiz-urquiza and Keyhani, 2013). The time of inoculation prior to ecdysis, and the length of the inter-moult period are important factors that may significantly influence susceptibility of the host insect to infection by entomopathogenic fungi (Butt and Goettel, 2000). Moulting may remove the penetrating fungus prior to the colonization of the insect, if it occurs shortly after inoculation (Vey and Fargues, 1977; Fargues and Rodriguez-Rueda, 1979; cited in Butt and Goettel (2000).

2.8.4.2 Solar radiation

Natural sunlight is one of the more important factors affecting survival of propagules under field conditions, the ultraviolet radiation-B (295-320nm) component being the most detrimental (Ignoffo, 1992), and is largely responsible for short persistence of mycoinsecticides in the epigeal habitat (Jaronski, 2010). Differences in susceptibility to UV radiation have been shown to exist between entomopathogenic fungal species and among strains within species (Benz, 1987; Ignoffo, 1992; Zimmermann, 2007; Jaronski,

2010). According to Ignoffo (1992), sunlight may directly inactivate entomopathogens through deletions, cross-linking, strand breakage, and/or formation of labile sites on DNA. On the other hand, the indirect effects of sunlight may involve generation of highly reactive radicals that in turn inactivate the entomopathogens (Ignoffo, 1992). Detrimental damage by UV light is caused by photoreactions of nucleic acids, proteins, lipids, and membranes (Tevini, 1993; cited in Vega *et al.* 2012). On the other hand, sub-lethal exposure to UV radiation can cause physiological or genetic alterations that result into reduced and delayed germination and reduced virulence (Braga *et al.*, 2001). Incorporation of UV protectants to conidial formulations has been reported to minimize the negative impact of UV radiation (Zimmermann, 2007).

2.8.4.3 Relative humidity

Relative humidity (RH) is another important environmental factor that affects critical stages of the infection process, namely, germination, penetration, and sporulation of entomopathogenic fungi (Benz, 1987; Inglis *et al.*, 2001; Meekes, 2001; Wraight *et al.*, 2007; Zimmermann, 2007). Some entomopathogenic fungal species are more dependent on high humidity than others with most requiring at least 95% RH at the insect surface for successful spore germination (Hallsworth and Magan, 1999). For instance, Damir (2006) found that spores of *B. bassiana* and *M. anisopliae* germinated at 15 – 30^{o}C and > 95% RH, and 20 – 30^{o}C and > 95% RH, respectively. On the other hand, some insects become infected at much lower humidity. To crown it, Jaronski (2010) suggests that dependence of fungal infection on humidity depends upon the insect and its ecology, especially in relation to the phylloplane and its microclimate.

2.8.4.4 Rainfall

The impact of rain on the persistence of fungal propagules on insects and on foliage has not been extensively studied (Inglis *et al.*, 2001; Wraight *et al.*, 2007). Moreover,

measurement of rain effects on conidial persistence in field environments is considered to be extremely difficult due to confounding variables, especially solar radiation (Wraight *et al.*, 2007). In a study to assess the effect of simulated rain on *B. bassiana* conidial removal, Inglis *et al.* (2000) recorded 89 to 95% removal of Colony Forming Units (CFU) from potato leaves, and 34 to 70%) from larvae and the majority of CFU were removed within the first 15 min of exposure. In another experiment involving different formulations, they noted that Conidia applied in sun-flower oil were not removed from potato leaves by exposure to 39 mm of rain in 30 minutes. In contrast, conidial persistence during this period was substantially reduced for conidia applied in water, a wettable powder containing clay, and an oil emulsion formulation applied at a low volume rate. Formulation plays a significant role in the retention of conidia on foliage exposed to rain, with oil-formulated conidia generally indicated to persist longer than aqueous formulations (Bateman *et al.*, 1993; Inglis *et al.*, 2000; Jackson *et al.*, 2009; Jaronski, 2010). Effects of rainfall could also have positive implications towards fungal epizootics. For example, rainfall can serve to dislodge and disperse conidia from substrates as well as aid in the dispersion of propagules (Inglis *et al.*, 2001). Inyang *et al.* (2000) however questions the importance of rain in the redistribution of inoculums as they found only very little inoculum in the soil outside the sprayed plots in a subsequent field experiment.

2.9 Formulation of entomopathogenic fungi

Formulation is one of the crucial steps in the development of biopesticides, which are widely known to be susceptible to biotic and abiotic factors. To maximize biocontrol efficacy, biopesticide development efforts must consider ecological and environmental factors (Jackson *et al.*, 2009; Jaronski, 2010). This is important especially in SSA where the prevailing high temperatures and UV radiation are more likely to negatively impact on the persistence of the fungi.

UV-protectants have been identified as one of the promising shields in providing protection to biocontrol fungi against UV radiation. For example, Leland and Behle (2005) noted that lignin-coated *B. bassiana* formulations provided the greatest protection against UV radiation although they were slower to kill the tarnish plant bug, *Lygus lineolaris* (Jackson *et al.*, 2009). They however noted that under field conditions where solar radiation significantly impacts mycoinsecticide efficacy, the improved persistence of lignin coating formulations may outweigh the negative effects of reduced pathogenicity. Clay and oil have also been identified as carriers providing protection against UV (Zimmermann, 2007). Generally, oil-formulated mycoinsecticides are reported to be more pathogenic than aqueous formulations or dry unformulated conidia, and formulating fungal conidia in oil improves their thermotolerance (Brooks *et al.*, 2004).

CHAPTER THREE

PATHOGENICITY OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* TO THE LEGUME POD BORER, *MARUCA VITRATA*, AND THE PERFORMANCE OF TWO CANDIDATE ISOLATES IN FOUR LIQUID CULTURE MEDIA

3.1 Summary

The legume pod borer, *Maruca vitrata*, is one of the most damaging insect pests of cowpea in sub-Saharan Africa (SSA). Twenty isolates of *Metarhizium anisopliae* and *Beauveria bassiana* were screened to select the most virulent isolate for managing the pest. Two most virulent isolates were selected and tested further against different developmental stages of *M. vitrata*. Their production potential in four liquid media was also assessed. *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 caused highest mortality of 91% and 81%, with LT_{50} of 1.8 and 1.7 days, and LC_{50} of 1.07×10^7 and 3.01×10^6 conidia ml^{-1}, respectively. The oil formulation enhanced fungal efficacy; the effect being more pronounced at the egg stage. Both ICIPE 18 and ICIPE 69 caused 100% mortality in males and females, while horizontal transmission of ICIPE 69 and ICIPE 18 by males reduced fecundity to 103 and 203 eggs/female, respectively, compared with 543 eggs/female in the control. Isolate ICIPE 69 yielded highest biomass and propagules in Jenkins-Prior and APU1 media, compared with ICIPE 18. Isolate ICIPE 69 holds a greater potential as a biopesticide for managing the East African population of *M. vitrata*, and Jenkin-Prior and APU1 media are appropriate for its mass production.

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3.2 Introduction

Cowpea (*Vigna unguiculata* L. Walp) is the most important grain legume crop in sub-Saharan Africa (SSA) (Adati *et al.*, 2008; Dugje *et al.*, 2009). It is a protein-rich grain that complements different starch staples and also provides fodder for livestock, improves soil by fixing nitrogen, and enhances household livelihoods by generating income in cash and alleviating poverty (Adati *et al.*, 2008; Dugje *et al.*, 2009). A plethora of insect pests attack cowpea, (Adati *et al.*, 2008; Oyewale and Bamaiyi, 2013) and can cause up to 100% yield loss if not effectively controlled (Dugje *et al.*, 2009). Among the different insect pests that constrain productivity of cowpea, the legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Pyralidae) is regarded as one of the most important (Dugje *et al.*, 2009; Ganapathy, 2010; Srinivasan, 2012). Damage is caused by the larvae which feed on floral buds, flowers, and pods. A single larva can consume 4 – 6 flowers before larval development is completed (Sharma, 1998). Varying losses in grain yield due to *M. vitrata* have been reported across different regions, but generally in the range of 20 – 100%. In Kenya yield losses of up to 80% are common on different varieties of cowpea (Okeyo-Owuor *et al.*, 1983).

Cowpea growers continue to rely largely on use of chemical insecticides to manage *M. vitrata* (Adati *et al.*, 2008; Sabo, 2015). However, farmers on several occasions misuse the insecticides causing adverse effects on humans, environment and biodiversity (Ton, 2000; Listorti and Doumani, 2001; Chopra *et al.*, 2005). Control failures have been reported in SSA and Asia, which have attributed to development of resistance to chemical insecticides in *M. vitrata* (Okeyo-Owuor *et al.*, 1983; Ekesi, 1999; Ulrichs *et al.*, 2001). Although some efforts have been made to exploit entomopathogens in managing *M. vitrata* (Ekesi *et al.* 2002), no convincing success has been documented. Moreover, there is documented evidence that suggests the existence of various populations and host races of *M. vitrata*. For example, variations in response of West Africa, Southeast Asia, and South Asia populations to synthetic sex pheromone lures of *M. vitrata* have been reported

(Downham *et al.*, 2004; Hassan, 2007; Schläger *et al.*, 2012; Srinivasan, 2012). It is probable that the difference in population may be responsible for the lack of efficacy of Nigerian isolates of EPF to the East African population of *M. vitrata*. There is the need therefore to identify potent isolates of EPF that are efficacious against different populations and races of *M. vitrata*.

Maruca vitrata is a difficult pest to manage. Adult moths deposit their eggs on flower buds, flowers, and leaves and, abscission scars also serve as oviposition sites (Sharma, 1998; Sharma *et al.*, 1999; Ganapathy, 2010). On the basis of this knowledge, Ekesi *et al.* (2002) suggested targeting the eggs that are scattered on the plant structures, with EPF. However, most insect eggs are resistant to infection by EPF, (Sáenz-de-Cabezón *et al.*, 2003) but some virulent isolates are able to bridge the chorion and cause disease on the eggs (Shi and Feng, 2004; Angel-Sahagún *et al.*, 2005; Wekesa *et al.*, 2006). Larvae of *M. vitrata* emerge from eggs in the early evening and throughout the night, they wander on plant surfaces feeding on tender plant stems, terminal shoots, and peduncles during vegetative growth, and on flowers as plants mature (Sharma *et al.*, 1999; Ganapathy, 2010). In the morning hours, the larvae return to their shelters. The older larvae on the other hand often bore into pods, and occasionally into peduncle and stems (Sharma *et al.*, 1999; Ganapathy, 2010). This behavior of feeding in hard-to-reach microhabitats makes them difficult to access with control agents, but the insects could also pick infection through secondary uptake of inoculums from plant surfaces.

A potentially important window of opportunity for management of *M. vitrata* is to target the young larvae with an evening spray of EPF when they emerge from eggs. By targeting early developmental stages, damages by subsequent, highly mobile and destructive fourth- and fifth-instars will be minimized. Timing the spray in the evening would also protect the EPF from the vagaries of ultraviolet radiation. Further protection of EPF and enhancement of their efficacy can be achieved through effective formulation (Jackson *et*

al., 2009). Oil-formulated mycoinsecticides are generally reported to be more effective than aqueous formulations as the oil enhances attachment of the conidia onto the insect's body surface and protects the propagules against the harmful effects of UV radiation (Inglis *et al.*, 2000; Brooks *et al.*, 2004; Jackson *et al.*, 2009; Jaronski, 2010).

Mass production is another important factor to consider in the selection of candidate EPF for biopesticide development (Goettel and Inglis, 1997; Jackson *et al.*, 2009). On solid media, Hyphomycetes produce differentiated asexual spores that are genetically stable, (Mangenot and Reisinger, 1976) while in submerged culture, they usually form single cells called blastospores through schizolytic separation at septa or by mechanical fragmentation of hyphae by yeast-like budding (Rombach, 1989). For different isolates, the formation of these structures either in solid and or liquid media, and the quantity and quality of the propagules that are produced for use against different insects, can be affected by the composition of the medium and culture conditions (Jackson *et al.*, 1997; Vidal *et al.*, 1998; Bae *et al.*, 2000).

To identify a potent fungal isolate for managing the East African population of *M. vitrata*, 14 isolates of *M. anisopliae* and six isolates of *B. bassiana* were screened for pathogenicity against the first instar larval stage of this pest. One of the most pathogenic isolates was also formulated in oil and tested against other developmental stages of the insect. Lastly, the effect of nutrition in four liquid media on concentration of propagules and biomass produced by the two most virulent isolates was also evaluated.

3.3 Materials and Methods

3.3.1 Insects

A colony of *M. vitrata* was started from a batch of larvae obtained from infested cowpea plants in a field at *icipe*'s Thomas Odhiambo Centre located on the shores of Lake

Victoria, Nyanza Province, Kenya. The field collected larvae were introduced to semisynthetic diet prepared following the procedure described by Onyango and Ochieng'-Odero (1993), and the culture was maintained in the rearing room in the rearing room at 26 – 30^{o}C, 60 – 75% relative humidity (RH), and a 12 L:12 D photoperiod (Figure 3.1). When larvae pupated, the pupae were carefully harvested from the diet using a pair of soft forceps onto petri plates (14 cm diameter) and later introduced into sleeved Perspex glass cage (30 x 30 x 30cm). Emerging adults were collected and introduced into another sleeved cage (40 x 40 x 40cm) made of an iron rod frame with a mosquito netting enclosure. They were fed on 10% sugar solution on balls of soaked cotton wool, and placed at the base of each cage. The sucrose solution was replenished after every 2 days. A potted cowpea seedling (approx. 2 weeks old) was introduced into the adults' cage to serve as oviposition sites for the female moths. The seedlings were destructively harvested and placed into well secured ventilated clear plastic cups and incubated at temperatures of 26 – 30^{o}C, 60 – 75% RH. Eggs hatched within 2 – 3 days. The hatched first instar larvae were then introduced into a freshly prepared diet as described above. The rearing cycles continued, and experiments commenced after the colony had gone through at least 10 generations. Field collected populations of *M. vitrata* were introduced into the laboratory colony after every 6 months to enhance genetic vigor.

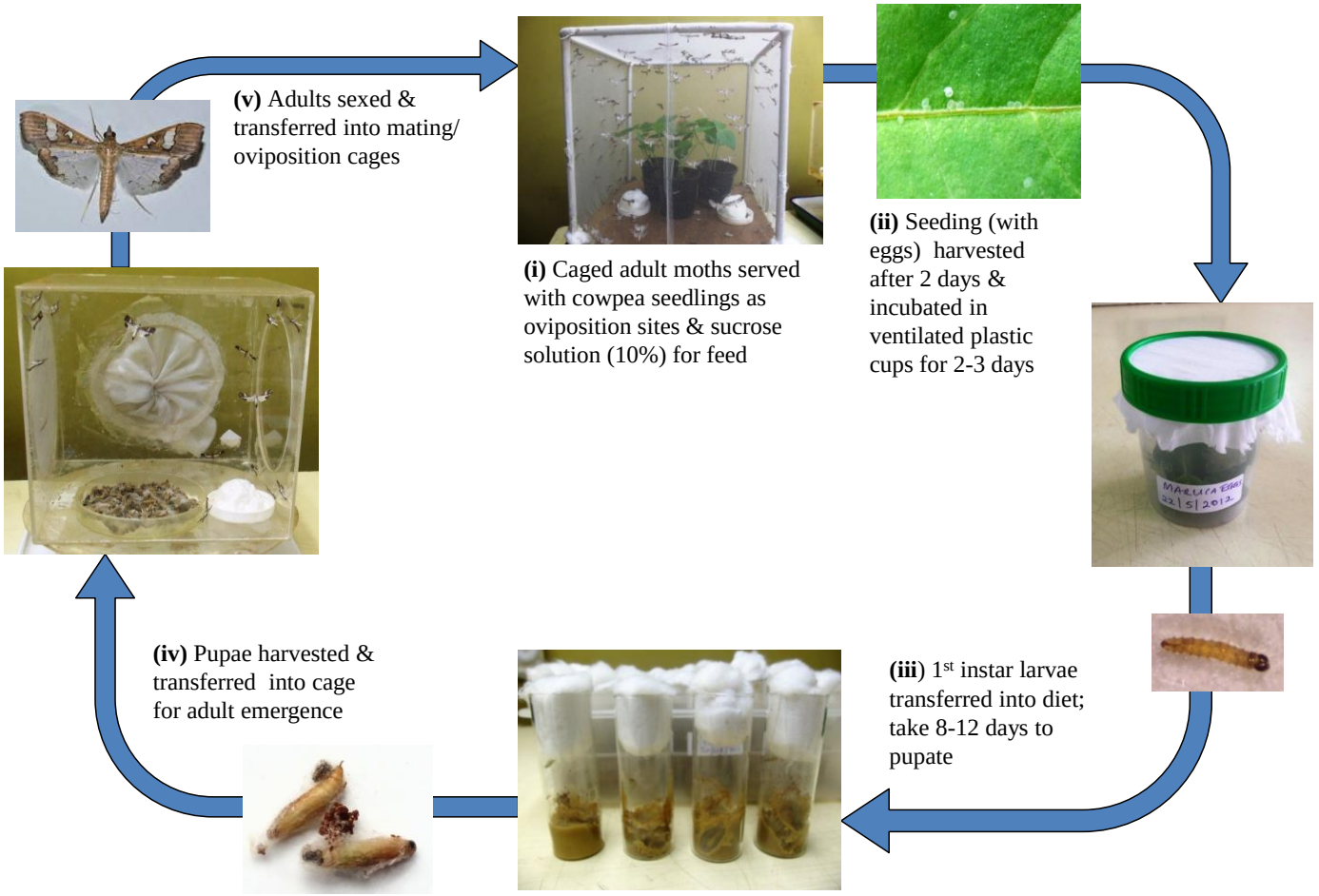
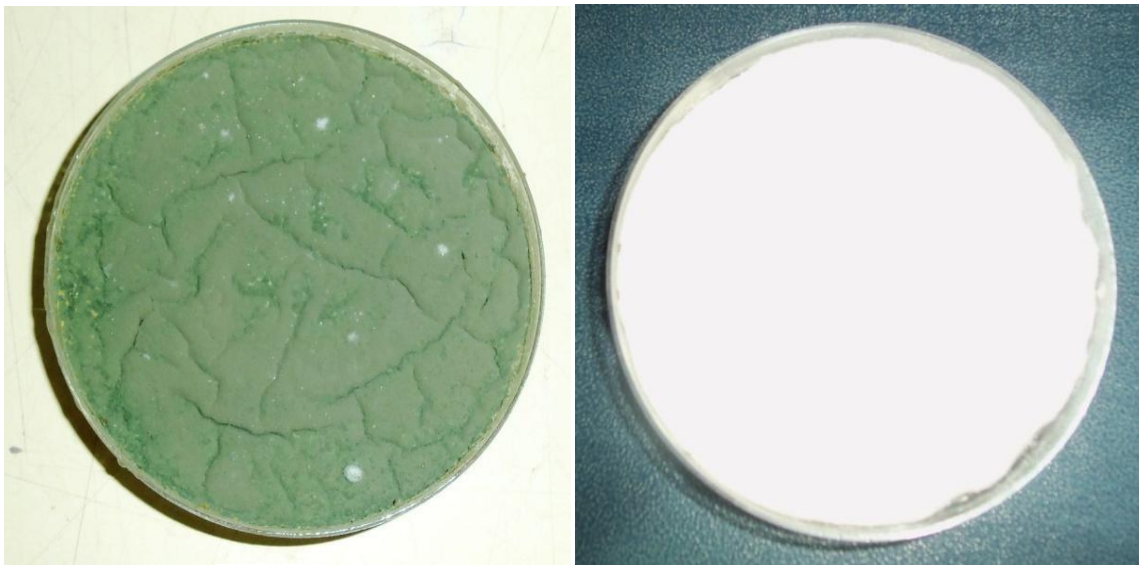


Figure 3.1 A graphical presentation of the procedure for rearing *M. vitrata* in the laboratory as described by Onyango and Ochieng'-Odero (1993) Photos: V. Tumuhaise.

3.3.2 Fungi

Fungal isolates used in this study were obtained from the *icipe*'s Arthropod Germplasm Centre, and included fourteen of *M.anisopliae* and six of *Beauveria bassiana*. Geographical locations and substrates from which these isolates were obtained are presented in Table 3.1. The isolates were chosen based on their ability to sporulate on Saboraud dextrose agar (SDA) medium upon retrieval from preservation; and ability of the spores harvested from sporulating cultures to cause mortality when passaged through second instar larave of *M. vitrata* and cause mycosis. Spores from mycosed cadavers were propagated on Saboraud dextrose agar (SDA) medium to obtain cultures for bioassays. Fungal suspensions were prepared by harvesting conidia from 2 – 3 weeks old sporulating cultures (Figure 3.2), and suspending them in 10-ml sterile distilled water containing 0.05% Triton X-100 in glass bottles with 10 glass beads (3 mm). Bottle contents were vortexed on a mechanical shaker (5 min, 10 cm of vertical travel and 700 oscillations per min) to produce a homogeneous conidial suspension. The conidia were then quantified following the procedure described by Inglis *et al.* (2012).

Conidial viability was assessed by spread-plating 0.1 ml of a suspension at a concentration of 3 x 10^6 conidia ml^{-1} on SDA medium. Inoculated plates were sealed with Parafilm membrane and incubated at 25 ± 2°C under complete darkness. At 18 h post inoculation, Lacto-phenol Cotton blue was added to terminate germination and also stain the spores to ease counting. Two sterile microscope cover slips were then placed on the surface of the 18 h old culture in each plate. Percentage germination was determined by counting 100 spores, categorized as germinated and non-germinated, under each cover slip on each Petri plate under the light microscope (x 400 magnification). A conidium was considered to have germinated if the germ-tube was at least twice the size of the spore (Inglis *et al.*, 2012). Each plate served as a replicate with four replications per fungal isolate originating from different plates.



A **B**

Figure 3.2 Sporulating cultures of selected entomopathogenic fungi used in the screening experiment: (A) *Metarhizium anisopliae*; (B) *Beauveria bassiana*. Photos: V. Tumuhaise.

Table 3.1 Fungal isolates tested against first-instar larvae of *M. vitrata* and their respective germination percentage 18 h post culturing on SDA media

Fungal spp. / Isolate	Year of isolation	Substrate	Country of origin
M. anisopliae			
ICIPE 69	1990	Soil	D.R. Congo
ICIPE 78	1990	*Temnoschoita nigroplagiata*	Kenya
ICIPE 18	1989	Soil	Kenya
ICIPE 62	1990	Soil	D.R. Congo
ICIPE 07	1996	*Amblyoma variegatum*	Kenya
ICIPE 665	2008	Soil	Kenya
ICIPE 655	2008	Soil	Kenya
ICIPE 315	2006	*Tetranychus urticae*	Kenya
ICIPE 31	2003	*Locusta migrotoria capita*	Madagascar
ICIPE 63	1990	Soil	D.R. Congo
ICIPE 20	1989	Soil	Kenya
ICIPE 30	1989	*Busseola fusca*	Kenya
ICIPE 22	1999	*Schistocerca gregaria*	Sudan
ICIPE 23	1998	*Ornithacris cavroisi*	Niger
B. bassiana			
ICIPE 280	2005	Soil	Mauritius
ICIPE 279	2005	Coleopteran larvae	Kenya
ICIPE 273	2006	Soil	Kenya
ICIPE 284	2005	Soil	Mauritius
ICIPE 669	2008	Soil	Kenya
ICIPE 647	2005	Soil	Mauritius

3.3.3 Primary screening of entomopathogenic fungal isolates against first larval instar of *Maruca vitrata*

3.3.3.1 Screening for pathogenicity

Each fungal isolate was assayed by dipping five freshly harvested cowpea flowers into 10 ml of a standard concentration of 1 x 10^8 conidia ml^{-1} for 30 s. After dipping, the flowers were placed on a paper towel for ca. 30 min to drain off excess suspension and thereafter transferred into clear plastic dishes (11 x 4cm) (Figure 3.3). A group of 30 first-instar larvae were then transferred onto the treated flowers and the dishes were covered with a screened/ventilated lid. Flowers in the control lots were dipped in sterile distilled water containing 0.05% Triton X-100. Insects were fed *ad libitum* on treated flowers, and were provided with fresh (surface sterilized and untreated) flowers daily as required. Larval mortality was recorded daily for 7 days. Dead insects were transferred to Petri plates lined with moist filter paper to facilitate the development of mycosis. Mortality due to fungal infection was confirmed by microscopic examination of hyphae and conidia on the surface of the cadaver. The screening bioassay was carried out in five batches, with each batch consisting of four fungal isolates and control, and each isolate was replicated four times.

3.3.3.2 Dose response bioassay

A dose response bioassay was conducted for the best two fungal isolates: *M. anisopliae* ICIPE 18 and ICIPE 69 which were selected based on cumulative mean mortality and LT_{50} values from the initial screening. The isolates were tested at five conidial concentrations: 1 x 10^4, 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8 conidia ml^{-1}. Five freshly harvested cowpea flowers were placed on Petri plates and sprayed with 10 ml of fungal suspension using the Burgerjon spray tower (Burgerjon, 1956). The Petri plates were lined with filter paper, before loading the flowers, to absorb excess suspension during spraying. Flowers in the control lots were sprayed with 10 ml of sterile distilled water containing 0.05% Triton X-100. For each isolate and dosage, 20 first-instar larvae of *M. vitrata* were

transferred onto the treated flowers in a plastic dish, and the dish covered with a ventilated lid. Insects were fed *ad libitum* on treated flowers within the first 24 h of treatment and further feeding protocol and monitoring of treated insects for mortality and handling of cadavers were done as described above. Each isolate and dosage under test was replicated four times.

Figure 3.3 Clear plastic dishes containing experimental *Maruca vitrata* larvae feeding on cowpea flowers treated with different concentrations of various fungal isolates. Inset (left top corner) is a larva of *Maruca vitrata.* Photo: V. Tumuhaise.

3.3.3.3 Assessment of conidia production on cadaver of first larval instar of *Maruca vitrata*

The two best isolates, *M. anisopliae* ICIPE 18 and ICIPE 69 were further evaluated for conidial production on first-instar larval cadavers. The larvae were exposed for 24 h to cowpea flowers treated with suspensions of isolate ICIPE 18 and ICIPE 69 at a concentration of 1×10^8 conidia ml^{-1}. At one day post inoculation, fresh untreated cowpea flowers were added as food for the larvae. Dead insects were collected 2 days post inoculation and incubated as described above. At 6 days post-incubation, five mycosed insects were collected and dried in an oven at 35^{o}C for 30 min, and thereafter transferred individually into 2-ml cryogenic tubes containing 1 ml of sterile 0.05 % Triton X-100. The tube was then vortexed for 5 min to dislodge conidia from the insect, and thereafter concentration of the resultant conidial suspension was determined as described above, and translated into estimate conidial yield by the cadaver. Each insect served as a replicate thus giving 5 replications per fungal isolate.

3.3.4 Susceptibility of egg, larval and adult stages of *Maruca vitrata* to different formulations of *Metarhizium anisopliae*

The aqueous and oil formulations of one of the most pathogenic isolate (*M. anisopliae* isolate, ICIPE 69) were tested against the egg, and four larval instars (i.e. first, second, third, and fourth) of *M. vitrata*. On the other hand, dry conidia were used in adulticidal activity assays. The aqueous formulated-fungus was prepared as described above (Section 3.3.2). On the other hand, the oil-formulated fungal suspension was prepared by emulsifying 1% (v/v) corn oil (Bidco Oil Refineries Ltd., Thika, Kenya), in sterile distilled water containing 0.05% Triton-X-100. For both formulations, the concentration was adjusted to 1 x 10^8 conidia ml^{-1}. Control for the oil formulation was prepared by emulsifying 1% oil in sterile distilled water (v/v), containing 0.05% Triton-X-100 while the control aqueous formulation constituted only sterile distilled water containing 0.05%

Triton-X-100. For the adulticidal activity assays, two *M. anisopliae* isolates (ICIPE 18 and ICIPE 69) were used in the form of dry conidia harvested from 2-weeks old cultures from SDA plates.

3.3.4.1 Ovicidal activity assay

Eggs oviposited by *M. vitrata* on potted cowpea seedlings within a period of 12 h were collected by destructively harvesting the leaves, and eggs counted under a dissecting microscope (x 16). Pieces of leaves carrying 20 eggs were cut off, placed in sterile Petri plate, and sprayed with 10 ml of either oil- or aqueous-formulated fungal suspension of *M. anisopliae* isolate ICIPE 69 using the Burgerjon spray tower. Eggs for the control groups were sprayed with 10 ml of the oil and aqueous control suspensions respectively, prepared as described above (Section 3.3.4). The treated eggs were thereafter separately transferred into ventilated clear plastic cups (6 cm diameter x 7 cm high), and incubated at 25 ± 2^{o}C. Four days post-treatment, the number of neonates (number of hatched eggs), were counted, and percent egg mortality computed. Four replicates were used per formulation.

3.3.4.2 Larvicidal activity assay

Treatment of larval instars was done by spraying cowpea flowers with 10 ml of the fungal suspensions prepared as described under 3.3.4 above, using the Burgerjon spray tower. Flowers for the control groups were treated with 10 ml of the oil and aqueous control suspensions, respectively. Insects were then released onto the treated flowers, as described under Section 3.3.3.1 above. Twenty insects were used for the first, second, and third larval instars, while 10 insects were used for the fourth instar larva due to limited supplies of the latter. Larval mortality was recorded daily for 7 days for all larval instars, starting 2 days after treatment. Dead insects were transferred to Petri plates lined with moist filter paper to facilitate the development of mycosis. Mortality due to fungal infection was

confirmed by microscopic examination of hyphae and conidia on the surface of the cadaver. Three replications were used per formulation and for each larval stage.

3.3.4.3 Adulticidal activity assays

Two bioassays were conducted involving the two *M. anisopliae* isolates, ICIPE 18 and ICIPE 69, and male and female adults of *M. vitrata*. The first assay assessed fungal effect as a result of direct application of spores to male or female moth. The second assay assessed fungal effects resulting from mixing directly inoculated males (as donors) and non-inoculated females (as recipients), to explore the possibility of horizontal transmission of inoculum from the former to the latter.

In the first bioassay, individual adult males and females were treated with dry fungal conidia following the procedure described by (Maniania *et al.*, 2011), with slight modification. Briefly, one-day old virgin adult males and females of *M. vitrata* were contaminated individually using small velvet coated plastic vials (1.5 cm diameter x 6 cm high). The insects were introduced into each of these vials that had been previously treated with 0.3 g of dry conidia of isolate ICIPE 18 or ICIPE 69 for 3 minutes to pick up spores. In the controls, dry spores inactivated by autoclaving were used. The treated insects were then transferred in groups of 10 males or females into sleeved Perspex cages (30 x 30 x 30cm), and provided 10% sucrose solution as food every 2 days. Adult mortality was monitored daily for 7 days. Dead insects were transferred to Petri plates lined with moist filter paper to facilitate mycosis. Mortality due to fungal infection was confirmed by presence of hyphae and conidia on the surface of the cadaver. Three replications were used per treatment (i.e. fungal isolate) for male and female moths.

In the second bioassay, one-day old males were individually treated as described above. Each treated male was then transferred into a sleeved Perspex cage (12 x 12 x 12cm) containing an uninfected one-day old virgin female. A fresh cowpea leaf mounted in a

glass vial containing water and plugged with moist cotton wool was introduced into the cage to serve as an oviposition substrate. Cotton wool soaked with 10% sucrose solution that was replaced after every two days was also provided for feeding. The cowpea leaf was removed from each cage after 24 h and eggs laid by each female were counted and a new leaf was introduced. Walls of the cage were also inspected and any oviposited eggs counted and removed to avoid repeated counting. Egg counting continued until the female died. Male and female longevity were also recorded. Each treatment had five couples with each couple serving as a replicate.

3.3.5 Performance of the selected *M. anisopliae* isolates ICIPE 18 and ICIPE 69 in liquid media

The nutritional composition of the four liquid media tested is presented in Table 3.2. The Adamek medium (Adamek, 1963), was originally described for producing submerged conidia of *M. anisopliae* while the Jenkins–Prior medium was developed for liquid production of submerged conidia of *M. flavoviride* (Jenkins and Prior, 1993). APU1 medium is a standard medium used in the *icipe* Arthropod Pathology Unit (APU), while APU2 medium is a slight modification of APU1 medium (Table 3.2).

For each medium, 100 ml were dispensed into 250-ml shake flasks and then sterilized and cooled (Figure 3.4). The flasks were then inoculated with a suspension of aerial conidia of the two *M. anisopliae* isolates, ICIPE 18 and ICIPE 69, to yield a final concentration of 1 x 10^6 conidia ml^{-1} culture. The flasks were then incubated on a reciprocal shaker (Innova® 44, New Brunswick Scientific, NJ**,** USA) (100 ± 2 travels min^{-1}) at 28 ± 0.5^oC. To estimate the concentration of the propagules, culture flasks were sampled at 72 h by removing 0.5 ml aliquots from each flask and counting at x 400 magnification using a Neubauer hemocytometer. At the same time the dry weight was determined by weighing 1 ml sample of the fresh weight matter and centrifuging at 3500 rpm at 5^oC (Medifuge centrifuge, Heraeus Sepatech GmbH, Osterode, Germany). The resulting precipitate was

washed twice with deionized water, weighed and later dried in an oven at 95^{o}C for 24 hrs, and biomass was estimated by determination of dry weight. Five replicated flask were maintained for each medium.

3.3.6 Statistical analyses

Mortality data were subjected to Abbott correction (Abbott, 1925), before analysis to correct for natural mortality. Data on concentration of propagules and biomass were Log_{10} transformed before ANOVA, as the data was highly skewed. The dose-mortality and time-mortality data were analysed by Generalised Linear Model (GLM) to generate LC_{50} and LT_{50} estimates, respectively along with slopes of the regression curves. In the case of dose-mortality data, GLM generated LC_{50} estimates along with their Standard Error (SE) values, and the latter were used to calculate 95% confidence intervals (CIs) [$LC_{50} \pm (1.96\times$ SE)]. In the case of LT_{50} estimation, the GLM analysis was run per replication and the resultant LT_{50} values and their respective slopes were subjected to ANOVA to generate means. Whenever treatment effects were found to be significant (P = 0.05), means were separated using Tukey's HSD test (P=0.05). For the primary screening bioassay, isolates that caused over 45% mortality were considered for LT_{50} estimation. On the other hand, LC_{50} estimates were subjected to pair-wise comparison and considered to be significant at the 5% level if their respective 95% CIs did not overlap. All analyses were done using R v2.15.1(R Development Core Team, 2008).

Table 3.2 Composition of four media (g l^{-1} distilled water) in shake-flask cultures of two *M. anisopliae* isolates

Ingredient	Ademek[1]	Jenkins-Prior[2]	APU1	APU2
Glucose	20	-	30	15
Sucrose	-	30	-	-
Corn steep liquor	15	-	-	-
Yeast extract	20	20	30	10
Waste brewer's yeast	-	-	-	20
Tween 80	4	4	-	-
Peptone	-	-	10	10
Streptomycin	-	-	1	1

[1](Adamek, 1963); [2](Jenkins and Prior, 1993)

Figure 3.4 Liquid media in 250 ml flasks which were inoculated with fungal conidia and incubated on the reciprocal shaker. Photo: V. Tumuhaise.

3.4 Results

3.4.1 Primary screening of *M. anisopliae* and *B. bassiana* isolates against first instar larvae of *M. vitrata*

Conidial viability of the test isolates ranged from 95 to 100% (Table 3.1); an indication that the bioassay measured true differences in virulence between the fungal isolates (Table 3.3). There were significant differences in mortality caused by the fungal isolates tested at the standard concentration of 1 x 10^8 conidia ml^{-1} (F = 10.6; d.f = 19, 60; P <*0.0001*). Among the 20 isolates tested, 2 isolates of *M. anisopliae*, ICIPE 18 and ICIPE 69, caused significantly higher mortality (81 and 91%, respectively) than other isolates (10 – 75%) at 7 days post inoculation (Table 3.3). These same isolates recorded the shortest LT_{50} values of 1.7 and 1.8 days, respectively (Table 3.3). Slopes of mortality curves for the most virulent isolates varied significantly with the best 2 isolates, ICIPE 18 and ICIPE 69 having the steepest slopes (Table 3.3). Higher doses caused significantly higher mortality than lower doses (Table 3.4). Isolate ICIPE 69 had a lower LC_{50} value (3.01 x 10^6 conidia ml^{-1}) compared with isolate ICIPE 18 (1.07 x 10^7 conidia ml^{-1}) (Table 3.5). The plot of Log-probit regressions for the two isolates reveals a steeper slope of the regression line for isolate ICIPE 69 than that of ICIPE 18, but both were significantly different from zero (P = 0.05) (Figure 3.5).

Table 3.3 Pathogenicity of *M. anisopliae* and *B. bassiana* isolates to first instar larvae of *Maruca vitrata*, 7 DAT, at a concentration of 1 x 10^8 conidia ml^{-1}

Fungal species/ Isolate	% germination ± SE	% mortality ± SE	LT_{50} ± SE	Slope (±SE)
M. anisopliae				
ICIPE 18	99.4 ± 0.4ab	91.2 ± 5.1 a	1.84 ± 0.1	6.31 ± 1.46 a
ICIPE 69	100.0 ± 0.0a	80.9 ± 9.1 ab	1.66 ± 0.48	4.18 ± 1.91 b
ICIPE 655	97.9 ± 0.2abc	75.0 ± 4.6 abc	1.05 ± 0.43	1.50 ± 0.46 b
ICIPE 20	98.0 ± 0.7abcd	63.9 ± 5.3 abcde	2.28 ± 0.20	1.56 ± 0.30 b
ICIPE 30	96.9 ± 1.0bcd	46.4 ± 4.7 cdefgh	na	na
ICIPE 22	96.1 ± 1.0cd	45.0 ± 3.8 cdefghi	na	na
ICIPE 07	98.8 ± 0.2abc	39.2 ± 4.3 defghij	na	na
ICIPE 315	98.2 ± 0.7abc	33.8 ± 8.9 efghij	na	na
ICIPE 62	98.8 ± 0.5abc	30.3 ± 7.1 fghij	na	na
ICIPE 665	98.6 ± 0.4abc	30.0 ± 4.8 fghij	na	na
ICIPE 63	97.5 ± 1.0abcd	29.8 ± 2.8 fghij	na	na
ICIPE 23	94.9 ± 0.8d	22.5 ± 11.1 ghij	na	na
ICIPE 78	100.0 ± 0.0a	12.2 ± 17.4 ij	na	na
ICIPE 31	98.0 ± 0.2abc	9.8 ± 3.5 j	na	na
B. bassiana				
ICIPE 284	98.3 ± 0.3abc	67.0 ± 1.4 abcd	2.94 ± 0.07	3.03 ± 0.17 ab
ICIPE 669	97.6 ± 0.6abcd	58.0 ± 8.3 bcdef	5.38 ± 1.01	3.86 ± 0.77 ab
ICIPE 273	98.3 ± 0.3abc	53.7 ± 4.4 bcdefg	4.46 ± 1.72	2.48 ± 0.99 ab
ICIPE 647	97.4 ± 0.8abcd	39.3 ± 5.0 defghij	na	na
ICIPE 280	99.2 ± 0.3ab	25.8 ± 6.3 fghij	na	na
ICIPE 279	98.6 ± 0.3abc	18.7 ± 5.9 hij	na	na

Means within a column followed by the same lower case letter are not significantly different by Tukey's HSD test ($P = 0.05$). [na] LT_{50} estimates for fungal isolates that never attained 50% larval mortality were higher than 7 days (the experimentation period), and were ignored.

Table 3.4 Cumulative mortality for selected isolates of *M. anisopliae* at different doses seven days after treatment

Dose (Conidia ml^{-1})	% mortality ± SE	
	Isolate ICIPE 18	Isolate ICIPE 69
1×10^8	75.3 ± 4.7aA	87.1 ± 6.7aA
1×10^7	34.5 ± 11.0bA	53.1 ± 8.2bA
1×10^6	34.1 ± 7.3bA	42.7 ± 9.8bcA
1×10^5	25.2 ± 2.6bA	9.7 ± 4.2dB
1×10^4	7.3 ± 1.6bA	16.7 ± 4.7cdA

Means within a column followed by the same lower case letter and within a row followed by the same upper case letter do not differ significantly by Tukey's HSD test ($P = 0.05$)

Table 3.5 LC_{50} values for selected isolates of *Metarhizium anisopliae* against *Maruca vitrata*

Fungal isolate	$[LC_{50}$ (CI)†$]x10^6$	Slope (±SE)[c]	Pr > χ^2	χ^2
ICIPE 18	10.66 (7.21 – 16.54)	0.74 ± 0.05***	<.0001	241.8
ICIPE 69	3.01 (2.29 – 4.23)	0.93 ± 0.05***	<.0001	336.64

[c] Significance codes at P=0.05: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

†CI = Confidence Interval

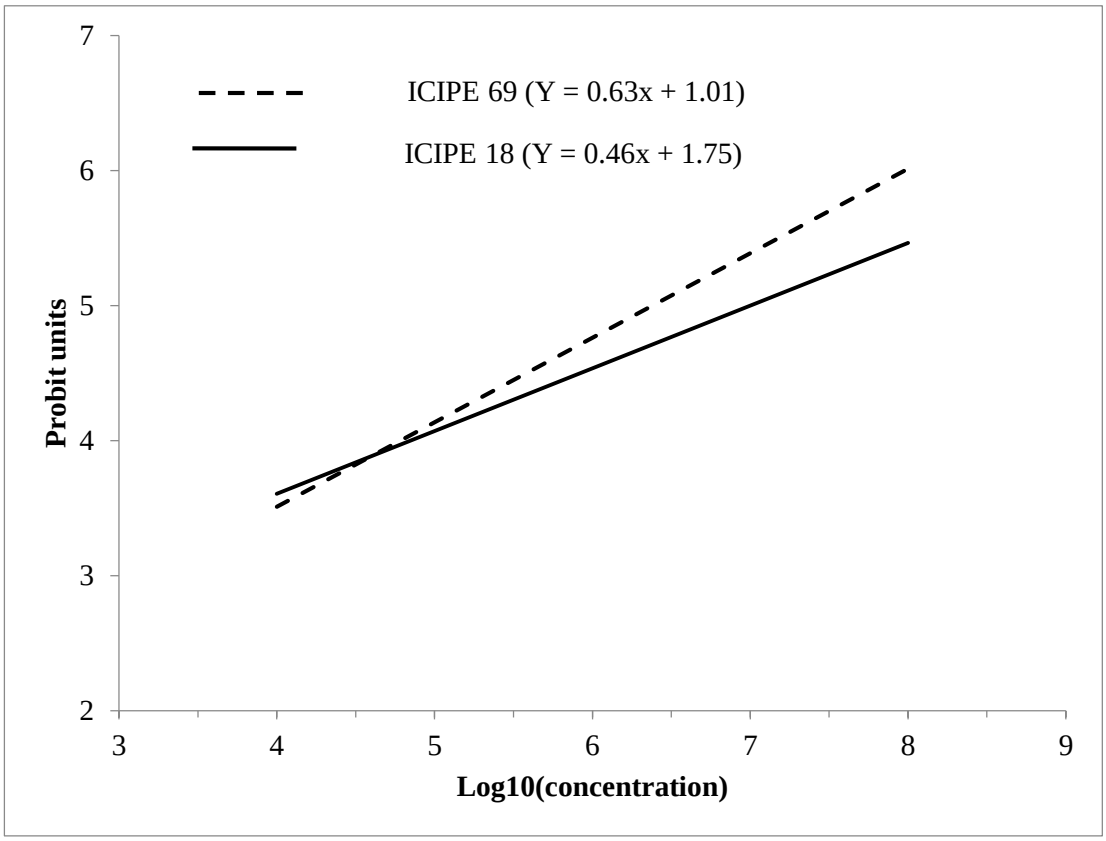


Figure 3.5 Log-probit regressions of mortality caused by *Metarhizium anisopliae,* isolates ICIPE 18 and ICIPE 69 on first instar larva of *Maruca vitrata*

3.4.2 Conidial production by *Metarhizium anisopliae* on *M. vitrata larval* cadaver

Isolate ICIPE 69 produced relatively more conidia (1 x 10^6 cadaver^{-1}) than ICIPE 18 (0.8 x 10^6), but the two were not significantly different (F = 0.23; df = 1; P = 0.6418) (Figure 3.6).

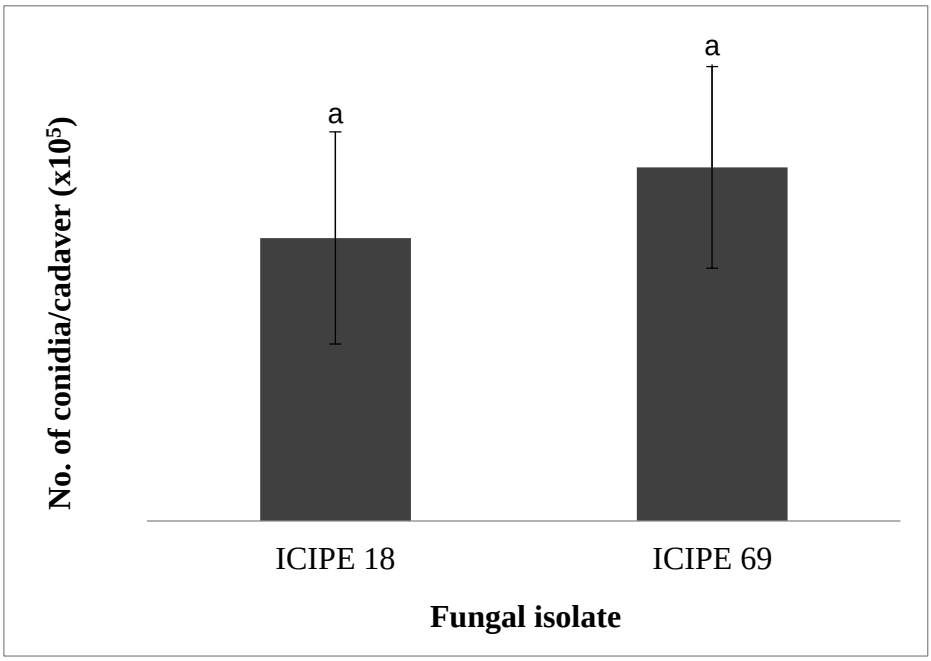


Figure 3.6 Mean conidial production on cadavers of first-instar larvae of *M. vitrata* treated with 1×10^8 conidia ml^{-1} of *M. anisopliae* isolates ICIPE 18 and ICIPE 69, six days post cadaver incubation.

3.4.3 Ovicidal and larvicidal activity of oil- and aqueous-formulated *Metarhizium anisopliae*

Developmental stage, as a factor, had a significant effect on mortality (F = 5.06; df = 4; P = 0.0055). On the other hand, no significant effect was observed due to formulation (F = 3.26; df = 1, P = 0.0861), and the interaction (i.e. developmental stage x formulation) (F = 1.57; df = 4; P = 0.2203). Significant differences were observed in mortality caused by the aqueous formulated ICIPE 69 (F = 4.60; df = 4, 10; P = 0.0229), with the highest mortality recorded among the first larval instar though not significantly different from the other larval instars but the egg stage incurred the lowest mortality. There were no significant differences in mortality among all the developmental stages treated with oil

formulated ICIPE 69 (F = 2.32; df = 4, 10; P = 0.1283), although the first and fourth larval instars had relatively higher mortality rates than the rest of the stages. The first larval instar had the lowest LT_{50} under both formulations – oil (3.36 days) and aqueous (3.08 days) (Table 3.6). Figure 3.7 represents 4th larvae of *M. vitrata* before, and after treatment with *M. anisopliae*.

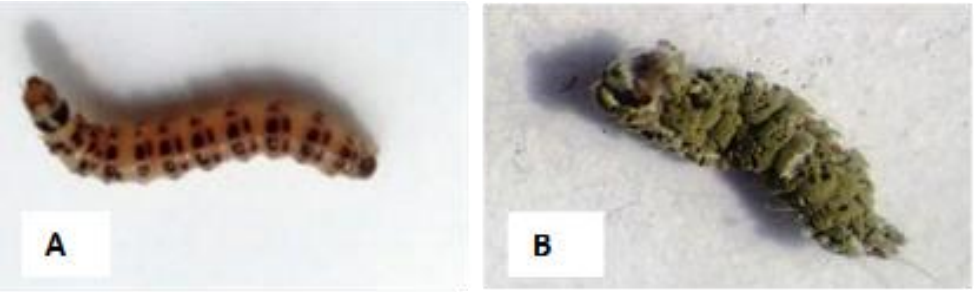


Figure 3.7 Fourth instar larvae of *Maruca vitrata*; **A**: Healthy larava before treatment with *M. anisopliae*; **B**: Cadaver of the 4h instar larva at 12 DAT (mycosed by *Metarhizium. anisopliae*). Photos: V. Tumuhaise.

Table 3.6 Pathogenicity of oil and aqueous formulated *Metarhizium anisopliae* isolate ICIPE 69 against the egg and larval stages of *Maruca vitrata*

	Oil formulation		Aqueous formulation	
Insect stage	% mortality ± SE	LT_{50} ± SE	% mortality ± SE	LT_{50} ± SE
Egg	66.7 ± 16.7a	ϕ	18.1 ± 9.2b	ϕ
1st instar larva	74.6 ± 6.5a	3.36 ± 0.43b	72.6 ± 5.9a	3.08 ± 0.50b
2nd instar larva	36.3 ± 19.5a	4.34 ± 0.71b	30.3 ± 4.5ab	15.6 ± 1.52a*
3rd instar larva	42.3 ± 3.9a	7.83 ± 0.85a	43.5 ± 15.6ab	5.02 ± 0.62b
4th instar larva	75.3 ± 4.4a	5.10 ± 0.06b	65.3 ± 13.8ab	5.33 ± 0.97b

Means within a column followed by the same lower case letter are not significantly different by Tukey's HSD test (P = 0.05). ϕ Eggs were excluded from LT_{50} analysis as egg mortality was assessed at once (i.e. 4 days post-treatment) as opposed to 7-days period for the larval mortality assessment. * LT_{50} for the aqueous formulation against the 2nd instar larva is an estimate beyond the 7 days mortality assessment period.

3.4.4 Adulticidal activity of *Metarhizium anisopliae*

Both isolates ICIPE 18 and ICIPE 69 caused 100% mortality to males and females (Table 3.7). Control mortality was at 3.3% and zero in males and females, respectively. Isolate ICIPE 69 had the shortest LT_{50} of 3.4 and 2.9 days in male and female *M. vitrata*, respectively. Significant differences in LT_{50} between ICIPE 69 and ICIPE 18 were only observed in female *M. vitrata* (F = 25.93; df = 1, 4; P = 0.0070) (Table 3.7). Females in the control groups and those paired with males treated with isolate ICIPE 18 started ovipositing 3 days after emergence while those paired with males treated with isolate ICIPE 69 started laying eggs 4 days after emergence (Figure 3.8). Number of eggs oviposited by females paired with fungus-inoculated males was lower than that observed

in the control group. In both control and treated groups, egg-laying sharply dropped after day 11, with no eggs recorded beyond day 14.

Fecundity in terms of eggs laid per adult female throughout its life time is presented in Table 3.7. Fungal isolate as a factor significantly affected the number of eggs laid by the adult female (F = 9.49; df = 2, 9; P = 0.0061). Isolates ICIPE 69 was significantly more effective in reducing daily oviposition (Figure 3.8), and ultimately the total number of eggs laid (up to 102.5 eggs/female), followed by isolate ICIPE 18 (232.8 eggs). The untreated control insects laid up to 542.8 eggs/female (Table 3.7). There was a significant difference in longevity of adult males directly treated with different isolates of *M. anisopliae* (F = 44.04; df = 2, 12; $P < 0.0001$). Isolate ICIPE 69 was significantly more effective in reducing male longevity (2.8 days), compared with isolate ICIPE 18 (7.0 days) while the untreated males lived significantly longer (15.6 days). On the other hand, no significant difference was noted among adult females infected indirectly through contact with inoculated males (F = 1.50; df = 2, 12; P = 0.2614). However, absolute values show that adult females treated with isolate ICIPE 69 lived relatively shorter (14.8 days) than those treated with isolate ICIPE 18 (15.6 days) while the untreated ones lived for 18.8 days (Table 3.7).

Table 3.7 Lethal (mortality) and sub-lethal (longevity and fecundity) effects of *Metarhizium anisopliae* against adult *Maruca vitrata*

	Virulence (% mortality and LT_{50})						
	Male		Female		Adult longevity (days)		
Fungal isolate	mortality ± SE	LT_{50} ± SE	mortality ± SE	LT_{50} ± SE	Male (donor)	Female (recipient)	Fecundity (eggs/female)
Control	3.3 ± 3.3b	*	0.0b	*	15.6 ± 1.4a	18.8 ± 1.2a	542.8 ± 114.0a
ICIPE 18	100.0a	3.8 ± 0.1a	100.0a	3.6 ± 0.1a	7.0 ± 0.9b	15.6 ± 0.7a	232.8 ± 51.4b
ICIPE 69	100.0a	3.4 ± 0.3a	100.0a	2.9 ± 0.1b	2.8 ± 0.4c	14.8 ± 2.6a	102.5 ± 23.1b

Means within a column followed by the same letter under each parameter, are not significantly different by Tukey's HSD test ($P = 0.05$)

* The control was excluded from the analysis for LT_{50} as mortality values for most replications in this group were zero.

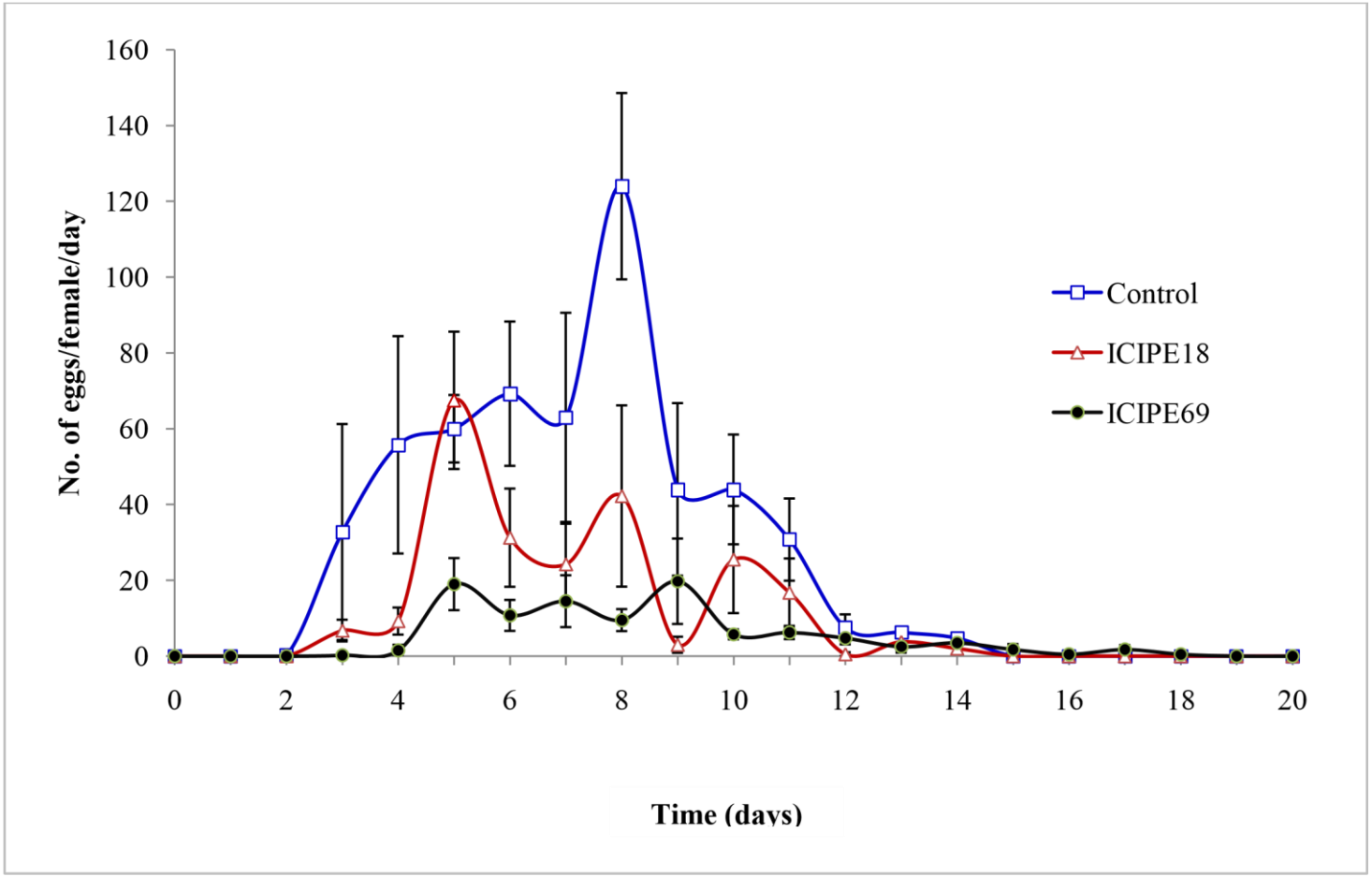


Figure 3.8 Oviposition pattern for uninfected female moths of *M. vitrata* paired with male moths infected with *M. anisopliae* isolates, ICIPE 18 and ICIPE 69.

3.4.5 Performance of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 in liquid media

There was a significant difference among the four media evaluated with respect to propagule production (F = 22.4; df = 3, 32; P = 0.0001) and isolates tested (F = 41.3; df = 1, 32; P = 0.0010). The interaction between media and isolate was also significant (F = 9.54; df = 3, 32; P = 0.0011). Among the four media tested, isolate ICIPE 18 produced significantly higher propagule concentration ($5.2 \pm 1.7 \times 10^7$ ml^{-1}) on APU1 than the other media. On the other hand, Jenkins-Prior ($2.6 \pm 0.4 \times 10^8$ ml^{-1}) and APU1 ($2.4 \pm 0.7 \times 10^8$ ml^{-1}) were the best media for ICIPE 69 compared to the other media. Comparison between the two isolates revealed a significantly higher concentration of propagules produced by ICIPE 69 on the two most effective media (APU1 and Jenkins-Prior), compared to ICIPE 18 (Table 3.8).

The amount of biomass produced by the two isolates after 72 h of oscillation significantly differed among the four media evaluated (F = 19.8; df = 3, 32; P = 0.0001) and the two isolates tested (F = 12.6; df = 1, 32; P = 0.0006) (Table 3.8). The interaction between media and isolate was also significant (F = 7.14; df = 3, 32; P = 0.00231). Among the 4 media, ICIPE 18 produced significantly higher biomass (38.7 ± 2.5 to 41.6 ± 3.1 mg ml^{-1}) on Ademek, Jenkins-Prior and APU1 than on APU2. On the other hand, biomass production by ICIPE 69 on Jenkins-Prior (68.3 ± 5.2) and APU1 (68.3 ± 2.1 mg ml^{-1}) were significantly higher compared to the other media. Overall, biomass produced by isolate ICIPE 69 on the best two media was significantly higher than that of ICIPE18 (Table 3.8).

Table 3.8 Propagule and biomass production of two isolates of *Metarhizium anisopliae* ICIPE 18 and ICIPE 69 after 72 h in four submerged culture media

	No. of propagules (ml^{-1} suspension) x 10^7		Biomass (mg ml^{-1} suspension)	
Medium	ICIPE 18	ICIPE 69	ICIPE 18	ICIPE 69
Ademek	0.2 ± 0.0bB	8.3 ± 0.3bA	40.3 ± 2.3aB	56.2 ± 3.6bA
Jenkins-Prior	0.2 ± 0.0bB	26.0 ± 4.0aA	41.6 ± 3.1aB	68.3 ± 5.2aA
APU1	5.2 ± 1.7aB	24.0 ± 7.0aA	38.7 ± 2.5aB	68.3 ± 2.1aA
APU2	0.1 ± 0.1bA	0.4 ± 0.08cA	20.5 ± 1.8bB	36.4 ± 10.6cA

Means within a column followed by the same lower case letter and within a row followed by the same upper case letter, under each parameter, do not differ significantly by Tukey's HSD test ($P = 0.05$)

3.5 Discussion

Results from the primary screening bioassay showed that all the fungal isolates tested against *M. vitrata* were pathogenic to the insect but their virulence varied greatly among the isolates; perhaps not surprising because interspecific and intra-specific variations in virulence between fungal species and isolates within fungal species are well documented for different species of insects (Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012; Tiago *et al.*, 2014).

Among the 20 isolates screened, results showed that *M. anisopliae* isolates ICIPE 18 and ICIPE 69 caused significantly higher mortality compared to the other isolates, and overall, isolates of *M. anisopliae* induced higher mortality than those of *B. bassiana*. Both *M. anisopliae* and *B. bassiana* are among the hyphomycetes reported to have wide host range

among invertebrates (Butt and Goettel, 2000) although some isolates can be host specific. Indeed, the two isolates of *M. anisopliae*, ICIPE 18 and ICIPE 69, identified in this study to be highly pathogenic to *M. vitrata* have also been reported to cause high mortality in other tropical insects. For example, from among 22 isolates screened, Ekesi *et al.* (1998) identified *M. anisopliae* isolate ICIPE 69 as one of the most pathogenic isolates to the legume flower thrips, *Megalurothrips sjostedti* (Trybom). It has also been found effective against the adult pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Migiro *et al.*, 2010). Similarly, ICIPE 18 has been found to be highly pathogenic to species of African tephritid fruit fly (Ekesi *et al.*, 2002b; Dimbi *et al.*, 2003), *L. huidobrensis* (Migiro *et al.*, 2010) and sandfly *Phlebotomus duboscqi* (Neveu-Lemaire) (Ngumbi *et al.*, 2011). This suggests that although widely distributed fungi such as *B. bassiana* and *M. anisopliae* could be relatively host-specific as pathotypes, some isolates such as the ones selected here can be effective against a broad range of insects, and could benefit from extension of labels to other crops upon registration and commercialization.

In this study, the lethal time to 50% mortality (LT_{50}) for the two most pathogenic isolates, ICIPE 18 and ICIPE 69, were short (1.7 and 1.8 days, respectively). Host death by entomopathogenic fungi is considered to be due to a combination of actions including production of toxins, physical obstruction of blood circulation, nutrient depletion and invasion of organs (Cory and Hoover, 2006; Inglis *et al.*, 2012; Shahid *et al.*, 2012; Vega *et al.*, 2012). On SDA, these isolates have been observed to exhibit rapid growth than the other isolates (Ekesi, pers. comm.), and this action probably assures faster penetration of the insect cuticle by the fungus. It is also possible that these isolates produce toxins that are responsible for the rapid death of the host, but this requires further investigation using chemical and or molecular techniques.

Susceptibility of most insects is dependent on spore dosage, and it is presumed that a threshold exists whereby a certain number of propagules are necessary to overcome the

host (Butt and Goettel, 2000; Wraight *et al.*, 2007; Shapiro-Ilan *et al.*, 2012). The slope of regression line for isolate ICIPE 69 was steeper than that of ICIPE 18, suggesting that the target insect will be more vulnerable over a given time with increasing doses of conidia (Ekesi *et al.*, 2002a) for isolate ICIPE 69 than ICIPE 18.

The bioassays involving the different developmental stages of *M. vitrata* revealed significant variability in their susceptibility to infection by *M. anisopliae*. Generally, adults were the most susceptible suffering up to 100% mortality at 7 days post infection, while larvae were more susceptible than eggs. Eggs were more susceptible to the oil-formulated than the aqueous-formulated *M. anisopliae*. Other studies have also reported differences in susceptibility among different developmental stages of other insect species. For example, Kirubakaran *et al.* (2013), reported variation in susceptibility of the adult and the second, third, and fourth instar larvae of the rice leaf folder, *Cnaphalocrocis medinalis* (Guenée) to oil-formulated *M. anisopliae* and *B. bassiana*. Additionally, Butt and Goettel (2000) also noted that not all stages in the life cycle of an insect are equally susceptible to fungal infection with pupal stages indicated to be often the most resistant stage and adults being the most susceptible.

Results of this study further show that first instar larvae were highly susceptible to fungal infection with over 70% mortality and had the shortest LT_{50} values of about 3 days. By targeting early developmental stages of *M. vitrata*, damages by subsequent, highly mobile and destructive matured fourth- and fifth-instars will be minimized. Upon egg hatch, first-instar larvae of *M. vitrata* wander around on the plants (Sharma, 1998; Ganapathy, 2010), and by so doing would pick up lethal doses of inoculums before they bore into the reproductive structures. Fungal infection in early instar larvae boring into flower buds and flowers will evidently benefit from the increased micro-climate humidity within the reproductive structures. A high relative humidity is an essential factor in the development of fungal infection (Wraight *et al.*, 2007; Jaronski, 2010; Vega *et al.*, 2012). The body size

and structure of the first instar present another opportunity for success; their cuticle is still delicate and therefore easy to degrade by EPF enzymes. Their small body size implies a large surface ratio that would therefore require less inoculum to colonise the hemocael and cause mechanical damage to internal organs. The low mortality recorded among the second and third instar larvae could be attributed to the physical and physiological fitness derived from feeding and therefore building immunity against infection (Butt and Goettel, 2000; Wraight *et al.*, 2007; Vega *et al.*, 2012).

Although no significant differences were observed in the mortalities among the larval instars treated with oil and aqueous formulations of *M. anisopliae,* generally, the oil formulation was relatively more efficacious than the aqueous formulation. Additionally, oil-formulated *M. anisopliae* had significantly higher ovicidal activity than the aqueous formulation. Oil-formulated mycoinsecticides are reported to show increased efficiency than aqueous formulations (Brooks *et al.*, 2004; Jackson *et al.*, 2009; Jaronski, 2010; Shahid *et al.*, 2012; Shapiro-Ilan *et al.*, 2012). This is attributed to the ability of oil to protect fungi against adverse environmental conditions, especially UV radiation, thus prolonging field persistence (Jackson *et al.*, 2009; Jaronski, 2010; Shahid *et al.*, 2012). Oil also improves adhesion of conidia to the insect cuticle and conveying the spores to the intersegmental membrane where infection is maximized (Jackson *et al.*, 2009).

The fact that the dry spores of the fungal isolates tested in this study were highly effective against adult *M. vitrata* causing 100% mortality with shorter LT_{50} values present promising management implications for this pest. For example, the conidia can be delivered through an auto-dissemination device combined with a pheromone lure. Autodissemination presents a cost effective window to reducing insect pest populations whereby insects are used to vector inoculum among conspecifics in the environment after they have been attracted and acquired the pathogen (Shapiro-Ilan *et al.*, 2012; Maniania and Ekesi, 2013). The potential of fungal auto-dissemination has been described for other

adult insect pests such as fruit flies (Dimbi *et al.*, 2013), and leaf miners (Migiro *et al.*, 2010). There is evidence that *M. vitrata* is attracted to synthetic sex pheromone lures (Downham *et al.*, 2004; Hassan, 2007; Schläger *et al.*, 2012; Srinivasan, 2012). Thus delivering the fungal conidia along these lures using an appropriate auto-inoculation device that ensures maximum spore pick-up by the insects and prolonged spore viability should highly impact on *M. vitrata* population. The short LT_{50} values especially for the fungus-treated female *M. vitrata* suggest that the female population could be significantly reduced before attaining the oviposition age.

The present study also revealed that *M. vitrata* females start laying eggs within 3 - 4 days after emergence, and continued for about 10 days beyond which few or no oviposition was recorded. This pattern concurs with previous reports by Sharma (1998), Sharma *et al.* (1999), and Sureja *et al.* (2010). Generally, daily oviposition by females paired with fungus-inoculated males was lower than that observed in the control group. In both control and treated groups, egg-laying sharply dropped after day 11, with no eggs recorded beyond day 14. This oviposition pattern resulted in significantly more eggs oviposited by females in the control group (543 eggs) than those paired with males infected with fungal isolates ICIPE 18 (233 eggs) and ICIPE 69 (103 eggs).

Although male longevity significantly varied between the control lots and those treated with fungal isolates ICIPE 18 and ICIPE 69, no significant differences were observed in female longevity. This could be attributed to the fact that males were directly inoculated with the fungal conidia compared to the female moths that were inoculated through secondary contamination by pairing them with the directly inoculated males. This cross-infection mainly occurs during the mating process when the two sexes come into contact but only at the genitalia. The observed result suggest that mating and other behavioural interactions between couples of health females and fungus-contaminated males do not result into passing on sufficient inoculums to the females that would cause mortality, but

rather only sublethal effects of reduced fecundity and longevity that compromise their ecological fitness. Sublethal effects of reduced longevity and fecundity due to entomopathogenic fungal infection are reported in other insect species such as the Colorado potato beetle, *Leptinotarsa decemlineata* (Fargues *et al.*, 1991), the pea leafminer *Liriomyza huidobrensis* (Migiro *et al.*, 2011), and the rice leaf folder *Cnaphalocrocis medinalis* (Kirubakaran *et al.*, 2013).

The nutritional conditions of the four media tested strongly affected growth and biomass production of the two isolates. Among the four media, the Jenkins-Prior and APU1 media were superior in terms of concentration of propagules produced with ICIPE 69 producing significantly higher concentrations of inoculums than ICIPE 18. The concentration of propagules produced by three strains of *M. anisopliae* ranged from 2.08 x 10^7 to 2.93 x 10^8 blastospores ml^{-1} (Kleespies and Zimmermann, 1992). In previous studies, the concentrations of propagules produced by two strains of *M. anisopliae* var *acridum* ranged from 1.4 – 2.4 × 10^8 propagules ml^{-1} in Adamek, Catroux, Jackson, and Jenkins–Prior media (Fargues *et al.*, 2002). However, with *M. anisopliae* var *acridum*, Jenkins and Prior (1993) reported concentration of 1.5 x 10^9 submerged conidia ml^{-1} after 7 days. The level of propagule production in this study is therefore within the range reported by other authors. Vidal *et al.* (1998) attributed higher yield of propoagules and biomass in Jackson medium to the higher quantity of sugar (80 g glucose 1^{-1}) in the medium. The reason for the high performance of Jenkins-Prior and APU1 media compared to the others in this experiment is unclear, but could also be attributed to the higher sugar source in the media. Indeed in *M. anisopliae*, not only does the amount of sugars affect productivity but also the nature of sugars as demonstrated by Kleespies and Zimmermann (1992) in which glucose and fructose were the best sugars compared to fructose or lactose for blastospore production.

Biomass production was highest in Jenkins-Prior, APU1 and Ademek compared with APU2 and overall, ICIPE 69 outperformed ICIPE 18 in terms of the quantity produced. On favourable media, biomass production in Hyphomycetes including *M. anisopliae* ranged from 14 mg ml^{-1} (Humphreys *et al.*, 1989) to as high as 60.3 mg ml^{-1} (Vidal *et al.*, 1998). Overall, among the two isolates evaluated in this study, ICIPE 69 produced higher propagule and biomass yield than ICIPE 18 on both Jenkins-Prior and APU1 and should be an ideal media for mass production of the isolate.

3.6 Conclusion

Metarhizium anisopliae isolates ICIPE 18 and ICIPE 69 are highly pathogenic against the egg, adult, and the first and fourth instar larval stages of *M. vitrata*. Oil formulation enhances the efficacy of these isolates against the egg and larval instars; the effect being more pronounced in the egg stage. Horizontal transmission of the fungal isolate from infected males results in sublethal effects of reduced fecundity and longevity, with isolate ICIPE 69 being more effective than ICIPE 18. Isolate ICIPE 69 also produces significantly higher concentration of propagules than isolate ICIPE 18 in two liqiuid media, Jenkins-Prior and APU1. *Metarhizium anisopliae* isolate ICIPE 69 therefore holds a greater potential as a biopesticide for managing the East African population of *M. vitrata*. Two liquid media, Jenkins-Prior and APU1, which are based on cheap locally available raw materials, should facilitate production of a low-cost biopesticide of *M. anisopliae* isolate ICIPE 69 that can be used by smallholder cowpea producers especially in East Africa.

CHAPTER FOUR

EFFECT OF TEMPERATURE ON GERMINATION, RADIAL GROWTH AND VIRULENCE OF *METARHIZIUM ANISOPLIAE* AGAINST THE LEGUME POD BORER, *MARUCA VITRATA*

4.1 Summary

The legume pod borer, *Maruca vitrata* is considered as one of the most important pests that constrain the production of legume crops in SSA. Entomopathogenic fungi (EPF) have been advocated as an environmentally acceptable alternative to synthetic insecticides in the management of *M. vitrata.* However, efficacy of EPF is generally influenced by biotic factors such as host insect, host plant and pathogen properties, as well as abiotic factors including, relative humidity, UV radiation, and temperature. This study was conducted to assess the effect of temperature on the germination, radial growth and virulence of two isolates of *Metarhizium anisopliae* against first-instar larvae of *M. vitrata*. Insect Life Cycle Modeling (ILCYM) was run and geographical information system (GIS) platform used to develop a map that predicts the geospatial variation in the efficacy of *M. anisopliae* strain ICIPE 69 against *M. vitrata*. The study revealed that there was no germination of *M. anisopliae* isolates ICIPE 69 and ICIPE 18 at 15^{o}C while germination was low at 35^{o}C. Optimum temperature for germination, radial growth and pathogenicity for isolate ICIPE 69 ranged between 25 – 30^{o}C, while that for ICIPE 18 ranged between 25 – 33^{o}C. The fastest growing isolate between 20 and 30^{o}C was ICIPE 69 while ICIPE 18 was the fastest growing isolate at 33^{o}C. Mortality of *M. vitrata* due to both isolates ranged between 56.0 – 91.6% across the different temperatures with the highest mortality at 25˚C (79.9 ± 6.1%) for ICIPE 69 and 30˚C (83.0 ± 3.3%) for ICIPE 18. The shortest LT_{50} for isolates ICIPE 18 and ICIPE 69 were recorded as 2.5 days and 2.2 days at, 33 and 25^{o}C, respectively. Geospatial prediction of locations where *M.*

anisopliae ICIPE 69 might be highly effective generated those location that have successfully been invaded *M. vitrata*. This study revealed that both isolates ICIPE 18 and ICIPE 69 have a broad temperature range of pathogenic activity against *M. vitrata* and therefore, great potential to be considered as biocontrol agent against the pest. The significance of these findings in relation to the management of *M. vitrata* is discussed.

4.2 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is mainly grown in the tropics and subtropical regions in the world, which include parts of Asia, Africa, Southern Europe, Southern United States, and Central and South America (Singh, 2005; Adati *et al.*, 2008; Dugje *et al.*, 2009) for vegetable and grains and to a lesser extent as a fodder crop. More than 11 million hectares are harvested worldwide, 97% of which is in Africa (Oyewale and Bamaiyi, 2013). However, the crop is considered too risky an investment by many growers because of the numerous pest problems associated with its production (Adati *et al.*, 2008; Dugje *et al.*, 2009; Ganapathy, 2010; Oyewale and Bamaiyi, 2013). The legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) is considered as one of the most important polyphagous pests of cowpea and other leguminous crops in SSA (Sharma *et al.*, 1999; Ganapathy, 2010; Srinivasan *et al.*, 2012; Ranga Rao and Rameshwar Rao, 2013; Srinivasan, 2014). Damage is caused by the larvae which feed mainly on floral buds, flowers and pods, with a single larva consuming 4 – 6 flowers before larval development is completed (Sharma, 1998). Cowpea yield losses of 20 – 80% have been reported in SSA due to this pest (Sharma, 1998; Ganapathy, 2010; Srinivasan *et al.*, 2012). The pest has also been reported to cause 20 – 30% yield loss in mungbean and yardlong bean in Asia (Sharma, 1998; Srinivasan, 2014).

Management of *M. vitrata* relies heavily on synthetic insecticides deployed by farmers, often to the exclusion of other methods of control (Agwu, 1997; Tamò *et al.*, 1997;

Sharma, 1998). However, due to the problems associated with excessive use of synthetic insecticides such as adverse effects on humans, the environment and biodiversity (Ton, 2000; Chopra *et al.*, 2005), and control failures that have been associated with the high level of resistance to the conventional insecticides (Okeyo-Owuor *et al.*, 1983; Ekesi, 1999; Ulrichs *et al.*, 2001), there is great need for more environmentally friendly options to control this insect pest.

Microbial control including the use of EPF is an acceptable alternative to synthetic insecticides in the management of a variety of insect pests, (Lacey and Siegel, 2000; Zimmermann, 2007; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012) and should be a suitable option for the control of *M. vitrata*. The efficacy of entomopathogenic fungi is however influenced by several factors including host and pathogen properties, as well as environmental factors (Cory and Hoover, 2006; Wraight *et al.*, 2007; Cory and Ericsson, 2010; Jaronski, 2010; Vega *et al.*, 2012). Temperature is considered as one of the most important factors that influence such crucial events as spore germination, host penetration and growth in the host, that determine the efficacy of entomopathogenic fungi (Fargues *et al.*, 1997; Li and Feng, 2009; Rangel *et al.*, 2010; Vega *et al.*, 2012).

Most entomopathogenic fungi have a wide range of temperature tolerances although deviations from generalized trends have been observed, across and within species (Fargues *et al.*, 1997; Davidson *et al.*, 2003). It has also been shown that temperatures optimal for infection, growth, and sporulation are usually much more restricted, generally in the range of 20 – 30^{o}C although some isolates can tolerate lower and higher extremes. *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) are reported to have their optimal temperatures for conidial germination and mycerial growth in range of 25 – 28^{o}C (Fargues *et al.*, 1992, 1997). These and several other studies demonstrate that temperature is a variable for which generalizations are difficult to make (Vega *et al.*, 2012). Therefore, in the process of

evaluating the ability of candidate fungus as a potential biopesticide, it is important to consider not only its virulence to the target insect but also suitability for environmental conditions occurring in the insect habitat (Cabanillas and Jones, 2009). On the other hand, predicting the global efficacy of any biocontrol agent in relation to temperature may represent an essential contribution to managing the risk of the pest to spread into new regions, which is particularly relevant in a world disturbed by the ongoing climatic changes (Williams and Liebhold, 2002; Kiritani, 2006; Thuiller *et al.*, 2006). Critical to commercial viability is the selection of isolates that are highly virulent to wide groups of targeted insect species under any environmental conditions (Bouamama *et al.*, 2010).

This chapter reports on the effect of constant temperature on germination, radial growth and virulence of two isolates of *M. anisopliae* against first-instar larva of *M. vitrata*. Constant temperatures of 15, 20, 25, 30, 33, and 35ºC were selected for this study to cover the range in which the target insect, *M. vitrata* thrives according to Adati *et al.* (2004). The study also reports the global prediction of the efficacy of *M. anisopliae* isolate ICIPE 69 against *M. vitrata.* The prediction model was applied to one isolate, ICIPE 69 because it is highly virulent a wide group of pest insects (Ekesi *et al.*, 2002b; Dimbi *et al.*, 2003; Migiro *et al.*, 2010; Ngumbi *et al.*, 2011). It has excellent production characteristics and does not degenerate upon successive sub-culturing on artificial media (SDA), and it is already commercialized as compared to *M. anisopliae* isolate ICIPE 18.

4.3 Materials and Methods

4.3.1 Insect culture

The stock culture of *M. vitrata* was established in the laboratory from field-collected larvae on infested cowpea fields at the *icipe*'s Thomas Odhiambo Centre, located on the shores of Lake Victoria at Mbita, Nyanza Province, Kenya. The larvae were reared on

semi-synthetic diet using the procedure described by Onyango and Ochieng'-Odero (1993) for over 12 generations before commencement of the experiments. Cages (30 x 30 x 40 cm) with one end to serve as sleeve were completely micro-screened with a mosquito net mesh. Thereafter, about 25 females and 25 males were introduced in each cage which contained 1 – 2 young cowpea plants (approx. 2 weeks old; 4 – 6 leaves) as oviposition substrate. Cotton wool soaked in 10% sugar solution was provided at the bottom corner of the rearing cages to serve as food. Female moths were allowed to oviposit for 2 days, after which the entire plants were destructively harvested and transferred into ventilated clear plastic cups (6 cm diameter x 7 cm high), and incubated at 26 – 30^{o}C, 60 – 75% RH to hatch. First instar larvae emerged on the surfaces of the leaf within 2 – 3 days. Larvae were reared on artificial diet until they pupated. The pupae were carefully harvested from the diet using a pair of soft forceps onto Petri plates (14 cm diameter) and later introduced into sleeved Perspex glass cage (30 x 30 x 30cm). The pupae were then transferred to sleeved Perspex glass cage (30 x 30 x 30cm) which contained cotton wool soaked in 10% sugar solution placed at the bottom corner of the rearing cages to serve as food for emerging adults. Adults emerged within 3 – 5 days. The culture was a room maintained at 26 – 30^{o}C, 60 – 75% RH under a photoperiod of 12 L: 12 D. The laboratory colony was rejuvenated with wild populations of *M. vitrata* every 6 months to maintain genetic vigor.

4.3.2 Fungi

Two fungal isolates, ICIPE 18 and ICIPE 69, isolated from soil in Democratic Republic of Congo (DRC) were used in this study. Start-up cultures were obtained from the *icipe* Arthropod Germplasm Centre and sub-cultured on SDA medium. Fungal suspensions were then prepared by harvesting conidia from the 2 – 3 weeks old sporulating cultures and suspending them in 10-ml sterile distilled water containing 0.05% Triton X-100 in 25-ml glass bottles glass bottles containing 10 glass beads (3 mm). The bottles were

vortexed for 5 min to produce a homogeneous suspension, and spores quantified following the procedure described by Inglis *et al.* (2012).

4.3.3 Effect of temperature on germination of spore of fungal isolates ICIPE 18 and ICIPE 69

To assess the effect of temperature on germination, 0.1 ml of conidial suspensions at a standard concentration of 3 x 10^6 conidia ml^{-1}, originating from different plates, were separately spread-plated on SDA media in Petri dishes (90 mm). Two sterile microscope cover slips were placed on each plate and the inoculated plates securely sealed with Parafilm membrane and incubated at constant temperatures of 15, 20, 25, 30, 33, and 35^{o}C under complete darkness. At 20 h post inoculation, Lactophenol Cotton Blue was added to each plate to halt germination and stain the spores for ease of visibility. Percentage germination was then determined by counting 100 spores from each plate at x 400 magnification. Each plate served as a replicate with four replications per treatment.

4.3.4 Effect of temperature on radial growth of fungal isolates ICIPE 18 and ICIPE 69

Conidial suspensions for the two fungal isolates, ICIPE 18 and ICIPE 69, at a standard concentration of 1x10^7 conidia ml^{-1} and originating from different culture plates were separately spread-plated on SDA media in Petri dishes (90 mm). Inoculated plates were then incubated at 25^{o}C under complete darkness for 3 days in order to obtain mycelial mats. The mats were cut from the culture plates into round agar plugs using an 8-mm diameter cork borer. Each agar plug was then transferred onto the centre of a fresh SDA media plate from which a similar size plug of media had been removed using the same cork borer. The plates with implanted mycelial plugs were then securely sealed with Parafilm membrane and incubated under complete darkness at constant temperatures of

15, 20, 25, 30, 33 and 35ºC. Radial growth was recorded daily for 12 days using two cardinal diameters, through two orthogonal axes previously drawn on the bottom of each Petri dish to serve as a reference (Figure 4.1). The experiment was replicated 4 times.

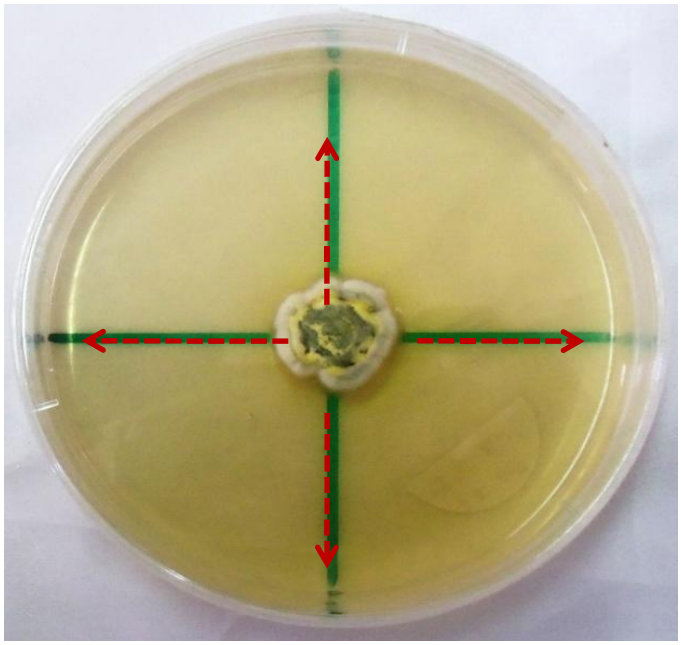


Figure 4.1 Radial colonization of Saboraud dextrose agar **(**SDA) medium by *M. anisopliae.* Photo: V. Tumuhaise.

4.3.5 Effect of temperature on virulence of fungal isolates ICIPE 18 and ICIPE 69

Fresh cowpea flowers were separately sprayed with 10 ml of conidial suspensions of the selected two fungal isolates (*M. anisopliae* ICIPE 18, and ICIPE 69) using the Burgerjon's spray tower (Burgerjon, 1956). The fungal isolates were tested at a standard concentration of 1 x 10^8 conidia ml^{-1} in 0.05% Triton X-100. Control lots were sprayed with sterile distilled water containing 0.05% Triton X-100. Treated flowers were transferred into clear

plastic dishes (11 x 4cm), and 20 first-instar larvae of laboratory-reared *M. vitrata* released onto the flowers. The treated larvae were maintained at constant temperatures of 15, 20, 25, 30, 33, and 35^{o}C, while recording larval mortality daily, starting 2 days post treatment, for 7 days. On each day after data collection, fresh surface sterilized flowers were provided as source of food, and insects fed *ad libitum*. Dead insects were transferred into Petri plates lined with moist filter paper, and sealed with Parafilm membrane to facilitate mycosis. Mortality due to fungal infection was confirmed by microscopic examination of hyphae and spores on the surface of the dead larvae 3 days post incubation. Each treatment was replicated 4 times.

4.3.6 Data analyses

4.3.6.1 Statistical analyses

Analysis of variance (ANOVA) was applied to percentage germination, growth, and mortality data. Growth was expressed as relative value (%) in relation to maximum speed of growth (Fargues *et al.*, 1997; Cabanillas and Jones, 2009) for each isolate at different temperatures before analysis. Percentage mortality (at 7 DAT) was also adjusted for natural mortality in controls (Abbott, 1925). The LT_{50} values were generated for each replicate using the Generalised Linear Model, and subjected to ANOVA and their means separated using Tukey's HSD test (P = 0.05). All analyses were done using R statistical software (R Development Core Team, 2008).

4.3.6.2 Development of temperature based model for the virulence of *Metarhizium anisopliae* isolate ICIPE 69 against *Maruca vitrata*

The relationship between temperature and mean proportional mortality of first instar *M. vitrata* across different fungal isolates were obtained by fitting several non-linear models. Parameter values were estimated by fitting the equation to the observed data from the

experiments at different constant temperature using a nonlinear optimization method, which find optimal parameter values that minimize the sum of square errors. The R statistical software was used to develop the code that was used for the task. The quadratic model emerged as the best for the available data points and is represented by the equation: $m(T) = b0 + b1x + b2x^2$, where "*m (T)"* represents the mean predicted mortality of first instar larvae of *M. vitrata* in relation to temperature, "x" represents the variable temperature while "b0", "b1" and "b2" are parameter constants.

4.3.6.3 Spatial simulation and mapping of the efficacy of *Metarhizium anisopliae* isolate ICIPE 69 against *Maruca vitrata*

The temperature data used for spatial simulations were obtained from WorldClim at a resolution of 10 minutes spatial resolution (www.worldclim.org). Simulations for predicting the mortality ***m*(T)** function in relation to temperature for each grid were based on average monthly temperatures. A computer program written in R (R Development Core Team, 2008) was used. First, the program extracted the average monthly temperature and organized them in 12 matrices based on their respective geographical coordinates (Longitude = column and Latitude = row). Secondly, a point object was created for each geographical coordinate and the mortality model was applied at each grid to estimate the percentage efficacy of the isolate. The output was then converted to American Standard Code for Information Interchange (ASCII) files; and transferred to ArcGIS 10.1 for mapping the estimated ***m*(T)** (mortality rate) values as an indicator for efficacy of *M. anisopliae* isolate ICIPE 69 for potential range separation and visualization.

4.4 Results

4.4.1 Effect of temperature on germination of *M. anisopliae* isolates ICIPE 18 and ICIPE 69

There was a significant difference in conidial germination between *M. anisopliae* isolates ICIPE 69 and ICIPE 18 across the different temperatures (F = 247.39; df = 11, 36; P<0.0001). No significant differences occurred among the fungal strains at 25^{o}C (F = 0.70; df = 1, 6; P = 0.4340). However, significant differences were observed at 20^{o}C (F = 40.17; df = 1,6; P = 0.0007), 30^{o}C (F = 7.11; df = 1,6; P = 0.0372), 33^{o}C (F = 10.01; df = 1,6; P = 0.0195), and 35^{o}C (F = 9.34; df = 1,6; P = 0.0223) . The optimum temperature for germination for *M. anisopliae* strain ICIPE 18 and ICIPE 69 were at 25˚C and 30^{o}C, respectively. There was no germination at 15^{o}C for both strains and germination at 35˚C was generally low for all the strains (Figure 4.2).

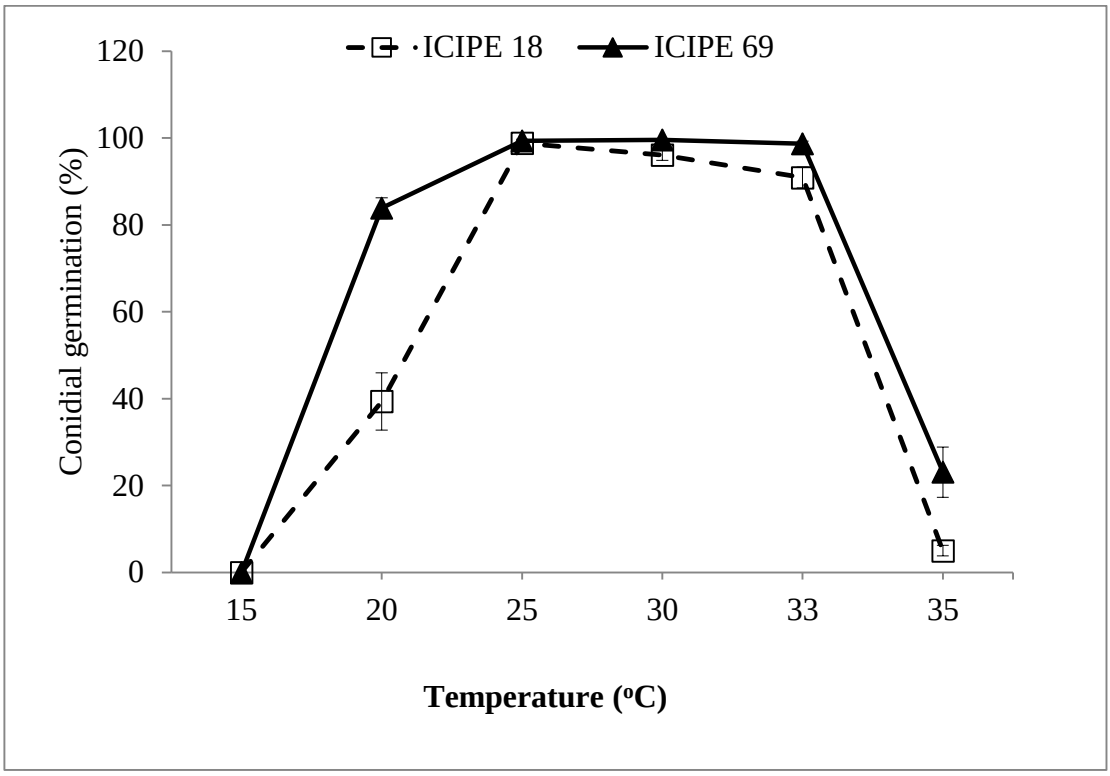


Figure 4.2 Effect of constant temperature on germination of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69

4.4.2 Effect of temperature on radial growth of *M. anisopliae* isolates ICIPE 18 and ICIPE 69

Growth occurred at all temperatures, but was slower at 15 and 35˚C as compared to 20, 25 and 30˚C (Figure 4.4 and Table 4.1). *Metarhizium anisopliae* isolate ICIPE 69 was the fastest growing strain at 15, 20, 25 and 30˚C. The optimum temperature for growth of isolate ICIPE 69 was at 30ºC and that of ICIPE 18 at 33ºC. There were no significant differences in growth rate between isolates ICIPE 18 and ICIPE 69 at 15ºC (F = 1.40; df = 1,6; P = 0.2815), 20ºC (F = 5.28; df = 1,6; P = 0.0613), and 33ºC (F = 4.23; df = 1,6; P = 0.0854). On the other hand, significant differences were observed at 25ºC (F =

95.49; df = 1,6; $P < 0.0001$), 30ºC ($F = 88.33$; df = 1,6; $P < 0.0001$), and 35ºC ($F = 197.47$; df = 1,6; $P < 0.0001$) (Table 4.1). Comparison of mean radial growth rates day^{-1} revealed that there were no significances between ICIPE 69 and ICIPE 18 at 15, 20, 25 and 30˚C (Table 4.1). There were highly significant differences in radial growths for each isolate across the different temperatures, i.e. ICIPE 18 ($F = 41.92$; df = 5, 18; $P < 0.0001$) and ICIPE 69 ($F = 80.01$; df = 5,18; $P < 0.0001$) (Table 4.1).

Table 4.1 Effect of temperature on growth rate of two isolates of *Metarhizium anisopliae* cultured on SDA medium for 12 days

Temperature (ºC)	Radial growth rate (mm day^{-1})	
	ICIPE 18	ICIPE 69
15	1.0 ± 0.0 cA	1.2 ± 0.1 cA
20	1.9 ± 0.3 bA	2.6 ± 0.2 bA
25	2.6 ± 0.1 aB	3.8 ± 0.0 aA
30	2.7 ± 0.1 aB	3.9 ± 0.1 aA
33	2.8 ± 0.1 aA	2.1 ± 0.4 bA
35	0.7 ± 0.0 cA	0.2 ± 0.0 dB

Means in the same column followed by the same lower case letters, and in same row followed by the same upper case letters are not significantly different ($P = 0.05$) by Tukey's HSD test.

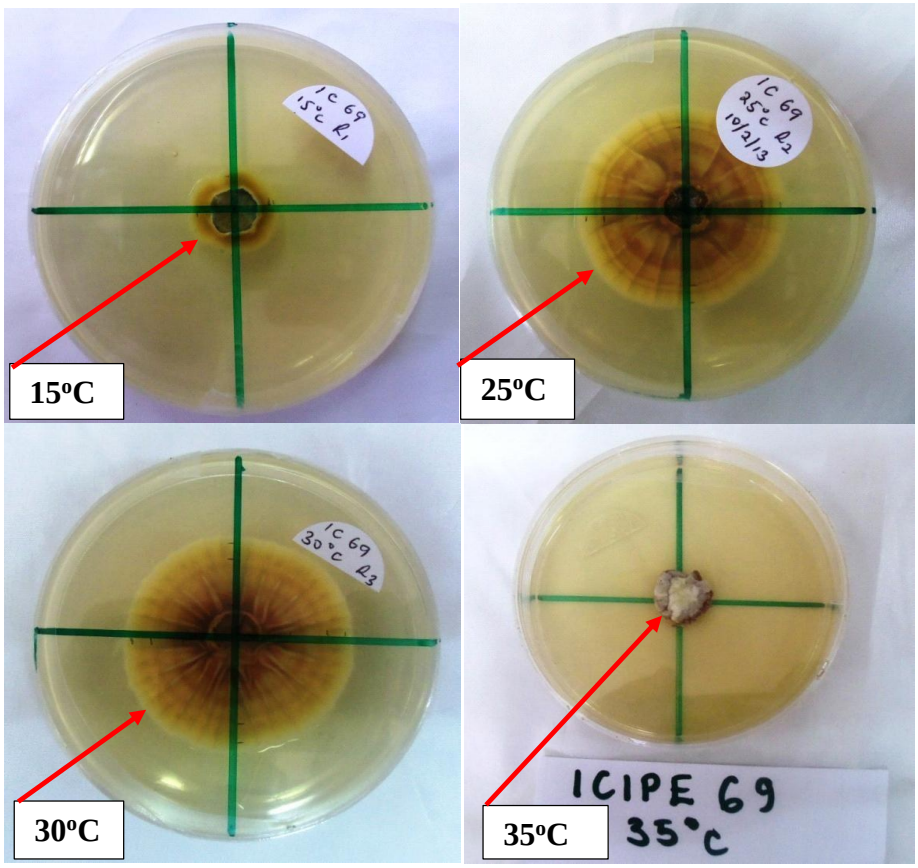


Figure 4.3 Radial growth recorded for *Metarhizium anisopliae* isolate ICIPE 69 at different temperatures, 18 days post inoculation. Red arrows indicate the growing edge of the fungal colony. Photos: V. Tumuhaise.

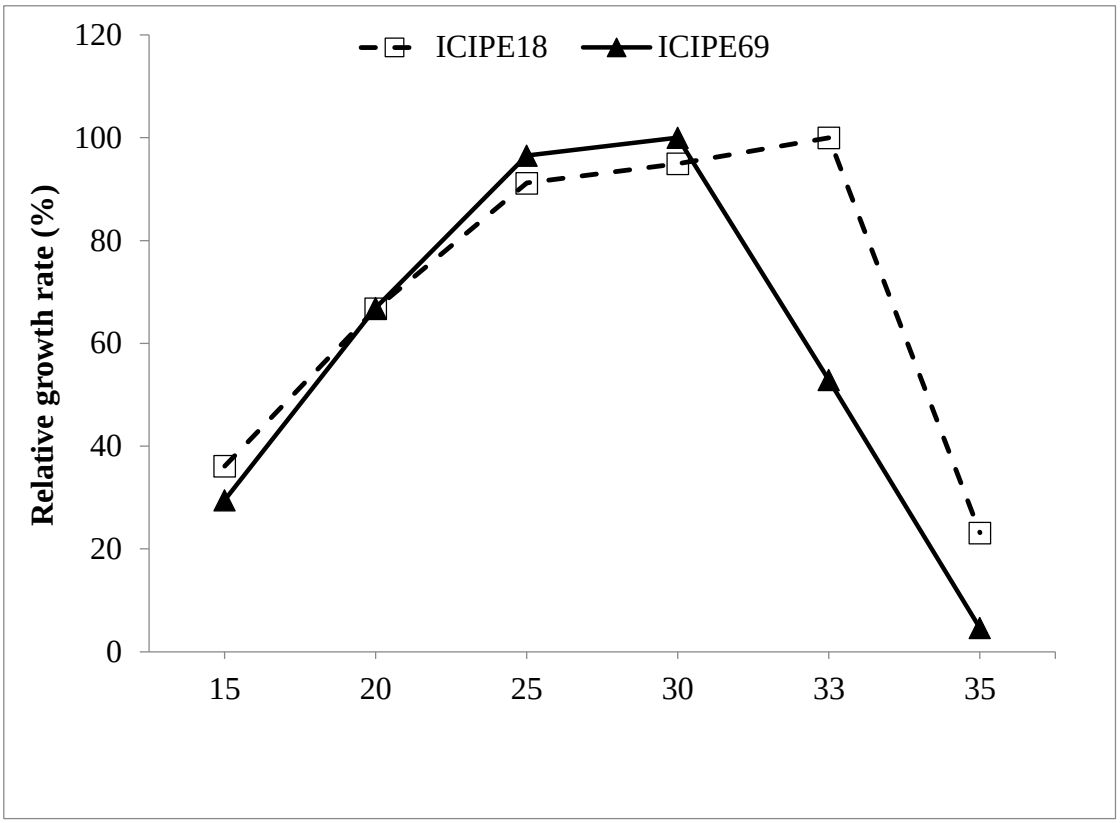


Figure 4.4 Effect of constant temperature on radial growth rate of two isolates of *Metarhizium anisopliae*

4.4.3 Effect of temperature on virulence of *M. anisopliae* isolates ICIPE 18 and ICIPE 69 against first instar larvae of *M. vitrata*

Mortality in the controls ranged between 0% (at 30^{o}C) and 30.4% (at 15^{o}C and 20^{o}C) (Table 4.2). All the isolates were virulent against *M. vitrata* across the temperature range of 15 – 33^{o}C causing mortality of 56.0 – 91.6%. Significant differences in virulence however were only observed at 30^{o}C with isolate ICIPE 18 causing a significantly higher mortality than ICIPE 69 (F = 7.85; df = 1, 4; P = 0.0487).

Table 4.2 Effect of constant temperature on virulence of two isolates *Metarhizium anisopliae* against first-instar larva of *Maruca vitrata*, 7 DAT

	% mortality ± SE		LT_{50} ± SE in days	
Temperature (°C)	ICIPE18	ICIPE 69	ICIPE 18	ICIPE 69
15	62.1 ± 12.4aA	56.0 ± 13.8aA	6.4 ± 1.15aA	5.3 ± 2.01aA
20	73.0 ± 9.0aA	63 ± 14.7aA	4.9 ± 0.84aAB	5.5 ± 0.84aA
25	65.0 ± 5.8aA	79.9 ± 6.1aA	2.4 ± 1.16aB	2.5 ± 0.94aA
30	83.0 ± 3.3aA	70.5 ± 3.0bA	2.8 ± 0.28aAB	3.3 ± 0.29aA
33	78.2 ± 7.9aA	69.4 ± 13.3aA	2.2 ± 0.32aB	5.6 ± 3.69aA

Means in the same row followed by the same lower case letters under each parameter, and in same column followed by the same upper case letters are not significantly different ($P = 0.05$) by Tukey's HSD test.

Table 4.2 also presents the speed with which the first instar larvae of *M. vitrata* succumbed to infection by *M. anisopliae* isolate ICIPE 18 and ICIPE 69 (i.e. LC_{50}). No significant differences were observed between the two isolates at each temperature. On the other hand, LT_{50} for isolate ICIPE 18 varied significantly across the six temperatures ($F = 4.75$; df = 4, 10; $P = 0.028$). There were no significant differences between LT_{50} values due to isolate ICIPE 69 across the six temperatures ($F = 0.55$; df = 4, 10; $P = 0.7052$). LT_{50} values for isolate ICIPE 18 ranged from 2.2 to 6.4 days with the shortest recorded at 33°C. This however did not differ significantly from the LT_{50} estimates at 25°C and 30°C (Table 4.2). On the other hand, LT_{50} estimates for isolate 69 ranged from 2.5 – 5.6 days, with the shortest at 25°C, although this was not significantly different from the LT_{50} values recorded at the other temperatures (Table 4.2).

4.4.4 Estimation of pathogenicity of *M. anisopliae* ICIPE 69 against *M. vitrata* using quadratic model

The relationship between temperature and mean mortality proportions of *M. vitrata* was modelled using non-linear regression models. The best fitting quadratic model (Figure 4.5) and parameter estimates for the model is presented in Table 4.3. The model predicted 25 – 30^{o}C to be the optimum temperature range for maximum efficacy of *M. anisopliae* isolate ICIPE 69 (Figure 4.5). Beyond the optimum temperature range of 25 – 30^{o}C, the model predicted a sharp decline in the efficacy, and at upper threshold temperature of 40^{o}C, the isolate was predicted not to be effective. The model predicted the lower threshold temperature close to 10˚C. The Akaike Information Criterion estimate for the model was -2.412 (Table 4.3).

Table 4.3 Parameter estimates for the best fitting quadratic model for predicting the mortality of first instar larva of *M. vitrata* across different temperatures

Parameter constants	Estimates	*t*-value	P ($\geq t$)
b0	$-0.003 \pm 6.641 \times 10^{-3}$	4.601	0.0100 *
b1	0.150 ± 0.034	4.453	0.0112 *
b2	-1.121 ± 0.381	2.94	0.0424 *
Akaike Information Criterion	-2.4120546		

* Significant at $P < 0.05$

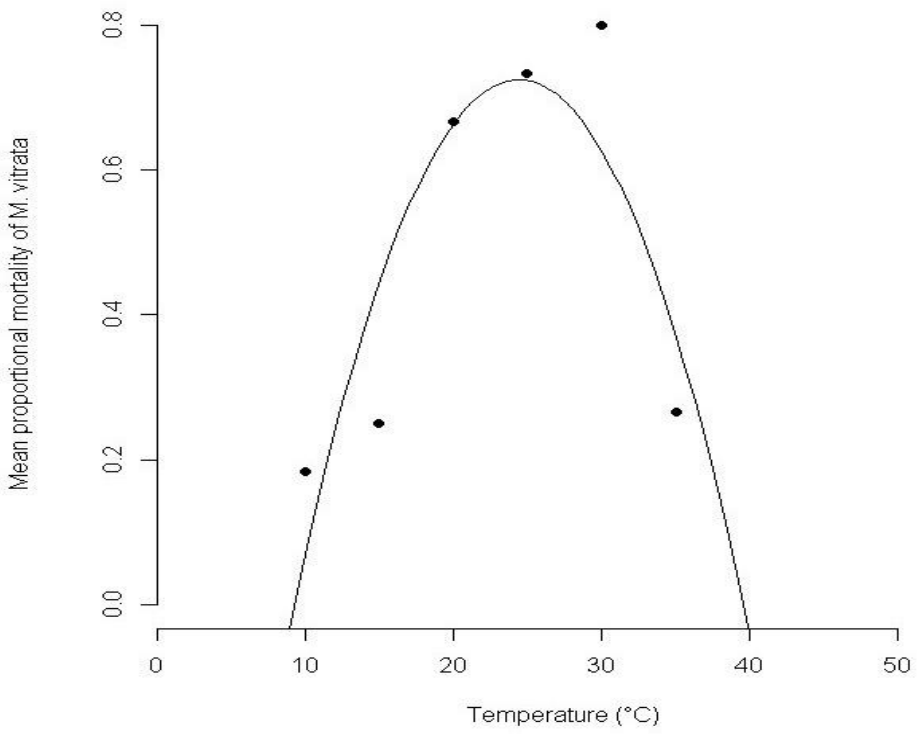


Figure 4.5 Observed and predicted mortality of first instar larvae of *M. vitrata* by *M. anisopliae* isolate ICIPE 69 in relation to temperature using quadratic model.

4.4.5 Spatial simulation and mapping of efficacy of *M. anisopliae* ICIPE 69

Spatial simulation and mapping of the efficacy of *M. anisopliae* ICIPE 69 revealed that the quadratic model predicted higher efficacy of the fungus in the tropics as compared to the temperate climates (Figure 4.6). The map predicted considerable variation in virulence of *M. anisopliae* ICIPE 69 between, and within, each country. The fungus was predicted to be ineffective in the tropical deserts of North Africa, Asia and in the temperate zones. The red areas on the map (Figure 4.6) are deemed by the model to be areas where *M. anisopliae* ICIPE 69 can be highly effective against *M. vitrata* and these suitable areas in the different regions strongly coincide with the sites where the pest has successfully invaded.

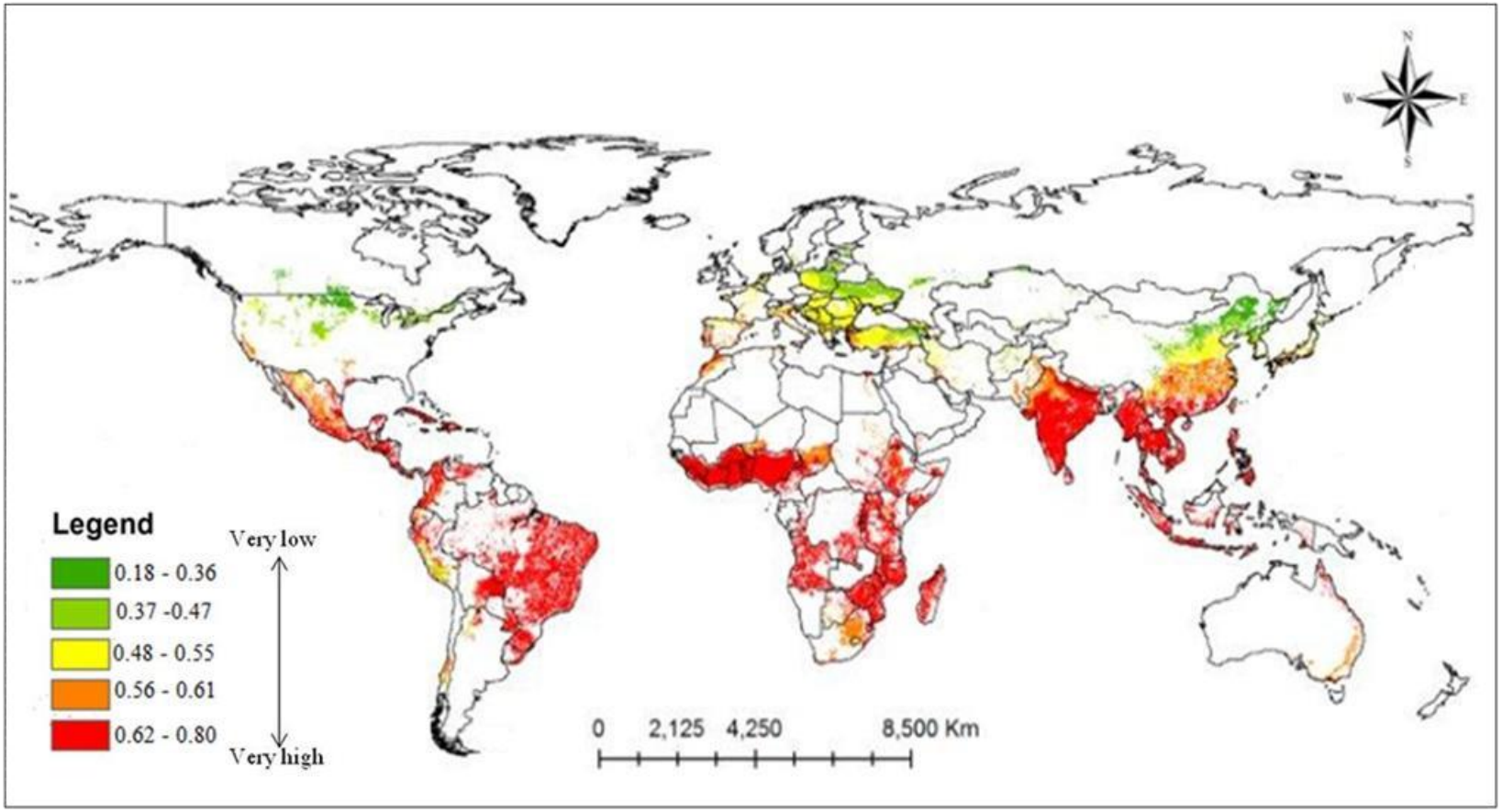


Figure 4.6 Global map predicting the efficacy of *M. anisopliae* isolate ICIPE 69 against first instar larvae of *M. vitrata* using the geospatial temperature data layer and the best fitted quadratic model. Five colours are used to indicate the strength of the prediction for each individual map pixel. White areas are deemed by the model to be areas where the fungus will be ineffective against the pest.

4.5 Discussion

Metarhizium anisopliae isolates ICIPE 18 and ICIPE 69 involved in study germinated at all experimental temperatures except 15^{o}C. For both isolates, the optimum temperature for germination was 25^{o}C. Fungal growth occurred at all temperatures, although extreme temperatures (15 and 35^{o}C) registered the lowest growth rates. Isolates ICIPE 69 and ICIPE 18 had optimum temperature for growth at 30 and 33^{o}C, respectively. The growth rates attained by isolate ICIPE 69 at the optimum temperature of 30^{o}C was highly comparable with that recorded at 25^{o}C. Similarly, growth rates for ICIPE 18 at the temperature range of 25 – 33^{o}C were highly comparable.

These results are comparable with those reported by Ekesi *et al.* (1999) for isolate ICIPE 69, and Dimbi *et al.* (2004) for isolate ICIPE 18 in their thermotolerance studies involving the legume flower thrips, *Megalurothrips sjostedti* (Trybom), and several African tephritid fruit flies, respectively. Most entomopathogenic fungi are reported to have a wide range of temperature tolerances, although temperatures optimal for infection, growth, and sporulation generally are in the range of 20 – 30^{o}C (Fargues *et al.*, 1997; Vidal *et al.*, 1997). Therefore, results from the present study concur with those reported in previous works. Tthermotolerance of entomopathogenic fungi is influenced by the geoclimatic origin of the fungal isolates, although cases of weak or no correlations between the geographical origin and thermal characteristics have also been reported (Fargues *et al.*, 1992, 1997). For example, fungal isolates originating from temperate regions had thermal thresholds as low as 8^{o}C while those from the tropics had their threshold in the range of 35 – 37^{o}C (Fargues *et al.*, 1997). The two fungal isolates tested in this study originated from the Democratic Republic of Congo with typical tropical temperatures.

From the virulence bioassay, both isolates, ICIPE 18 and ICIPE 69 were virulent against *M. vitrata*, causing mortality of 56.0 – 91.6% across the test temperatures (15 – 33^{o}C).

Optimum temperatures for infection by isolate ICIPE 18 was 30ºC while that of ICIPE 69 was 25ºC. Mortality due to these isolates generally increased with increase in temperature up to the respective optima of 30ºC and 25ºC for isolate ICIPE 18 and ICIPE 69, respectively. The optimal temperatures for germination, growth and virulence established from this study fall within the reported thermal thresholds for the development of *M. vitrata*. According to Adati *et al.* (2004), lower thermal thresholds for the development in the egg, larval and pupal stages of *M. vitrata* were 10.5, 10.0 and 10.9°C. On the other hand, the upper threshold was estimated to be in the range of 29.3 – 31.9°C depending on the developmental stage (Adati *et al.*, 2004).

Time to 50% mortality (LT_{50}) for isolates ICIPE 18 and ICIPE 69 was shortest at 33ºC and at 25ºC respectively. These temperatures closely correspond with optimum temperatures for growth, suggesting a close relationship between growth and virulence for these isolates. It is documented that host infection by fungal pathogens is positively correlated with temperature, but the optimum temperature for fungal development and that of disease development are not necessarily the same (Fargues *et al.*, 1992; Inglis *et al.*, 2001). For example (Doberski, 1981) found that positive infection of elm bark beetles inoculated with *B. bassiana* occurred at 2ºC and 10ºC for *M. anisopliae.* This therefore could explain the lack of perfect match between the optimum temperature for fungal growth and that of fungal virulence observed in this study. Apart from having a direct effect on the pathogen, temperature may exert its effect indirectly by moderating growth rate of the insect host. Thus temperatures that facilitate rapid growth of the insect would cause rapid moultig that may removal of the penetrating fungus before successful infection of the insect especially if it happens soon after inoculation (Butt and Goettel, 2000).

The quadratic model fitting the relationship of temperature to infectivity indicated that mortality of *M. vitrata* increased significantly as temperature increased up to an optimum temperature range of 25 – 30ºC for *M. anisopliae* ICIPE 69, beyond which the mortality

reduced. The model predicted a sharp decline in efficacy with a lower threshold close to 10ºC. The use of non-linear regression models to predict the efficacy of fungi in relation to temperature has the potential for identifying where and when the bio-pesticide could be used effectively and hence, for assisting in the development of optimum used strategies against insect pest infestations (Klass *et al.,* 2007). This is very valuable during the implementation of biological control program given the expense of field trials and difficulty in assessing mortality against highly mobile species especially in small-scale preliminary application trials (Klass *et al.,* 2007). The geo-spatial *M. anisopliae* ICIPE 69 efficacy maps shows that the predictions from the quadratic models indicate higher efficacy in the tropics than the temperate regions, coinciding with areas where *M. vitrata* has successfully invaded (CABI, 1996). The prediction results for efficacy of *M. anisopliae* ICIPE 69 from this study are in accordance with other studies, which have demonstrated that *M. anisopliae* has considerable potential for control of locusts and grasshoppers, and has been tested extensively throughout Africa, Australia, parts of Europe and Latin America (Thomas *et al.*, 2000; Lomer *et al.*, 2001). The predictions are also in line with the reports of Klass *et al.* (2007), who predicted lower efficacy of *M. anisopliae var. acridum* for control of locust in the Northern South Africa.

4.6 Conclusion

Metarhizium anisopliae isolates ICIPE 69 and ICIPE 18 are virulent against *M. vitrata* over a wide temperature range. These isolates can thus be further developed and used within the framework of IPM programs in the field. This study has also developed a useful modeling tool to help explore and evaluate the variability in performance of *M. anisopliae* isolate ICIPE 69 for control of *M. vitrata,* and has provided a graphic representation of how virulence can dramatically vary throughout the world. This study strongly demonstrates the potential of *M. anisopliae* isolate ICIPE 69 as candidate biological control agent for *M. vitrata* in vegetable legumes especially in the tropics.

CHAPTER FIVE

EFFECT OF HOST PLANT ON GERMINATION, PERSISTENCE AND VIRULENCE OF *METARHIZIUM ANISOPLIAE* AGAINST THE LEGUME POD BORER, *MARUCA VITRATA*

5.1 Summary

The legume pod borer, *Maruca vitrata* is one of the most limiting factors in legume production, infesting at least 37 legume plants, with yield losses of 20 – 80% reported across different parts of the world. Host plant morphological and biochemical properties can affect the effectiveness of entomopathogens, which are considered a safer alternative to synthetic insecticides. This study aimed at assessing the interaction between three plants and two isolates of *Metarhizium anisopliae* previously found virulent against *M. vitrata.* Cowpea induced highest virulence of isolate ICIPE 18 (77.5%) and lowest on beans (36.6%). Virulence of ICIPE 69 was highest on beans (95.7%), and least on *C. cajan*. No differences were observed in conidial germination. However, exposition of spores to flowers of *C. cajan* resulted in significantly fewer CFU (7 CFU) compared with other treatments and control (25.0 – 31.0 CFU). Fungal persistence was affected by plant, fungal isolate, and time factors resulting in more than 90% reduction in CFU at 3 days after spraying. The results suggest that host plant can significantly alter the efficacy of fungal-based biopesticide and interaction between the target host plant and the fungal isolate should routinely be investigated as part of the biopesticide development process.

5.2 Introduction

The legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) is considered as one of the most important legume pests because of its extensive host range, destructiveness, and distribution (Sharma, 1998; Margam *et al.*, 2011). It has been observed to feed on up to 45 host plants including two non-leguminous hosts (i.e.

Sesamum sp. and *Hibiscus* sp.) in tropical Asia and sub-Saharan Africa, (Srinivasan, 2014) causing yield losses of 20 – 80% reported across different parts of the world (Rathore and Lal, 1998; Sharma, 1998)). Several entomopathogens have been reported attacking *M. vitrata,* (Otieno *et al.*, 1983; Odindo *et al.*, 1989; Okeyo-Owuor *et al.*, 1991; Huang *et al.*, 2003) thus suggesting a possibility of harnessing the potential of these agents as alternatives for chemical pesticides in the management of this pest. Fungi are unique from other entomopathogens in that they infect their hosts primarily through the external cuticle, although a few taxa (e.g. *Culicinomyces*) are able to invade through the alimentary canal (Inglis *et al.,* 2001). Earlier laboratory studies demonstrated that *M. vitrata* is susceptible to *Metarhizium anisopliae* (Metschnikoff) and *Beauveria bassiana* (Balsamo) (Ekesi *et al.,* 2002a).

Morphological and biochemical properties of plants associated with defense against insect pests and phytopathogens have been reported to play a role in insect-pathogen interactions (Butt and Goettel, 2000; Poprawski *et al.*, 2000; Ekesi *et al.*, 2000a; Klingen *et al.*, 2002; Cory and Hoover, 2006; Cory and Ericsson, 2010; Vega *et al.*, 2012). Plant effects may include for example, plant exudates affecting the conidia directly; herbivore-induced plant volatiles affecting sporulation or germination; and leaf modifications of microclimate and thus spore germination (Cory and Ericsson, 2010). Others include plant quality, either allelochemicals or nutrients, altering insect condition (e.g. immunity) and thus disease resistance, or nutritional quality altering insect growth rate, which might alter the exposure of the insect to fungal entomopathogens (Butt and Goettel, 2000; Cory and Ericsson, 2010). Some reported examples of entomopathogenic fungi affected by host plant factors include *M. anisopliae* (Ekesi *et al.*, 2000a; Klingen *et al.,* 2002); *B. bassiana* (Gatarayiha *et al.*, 2010; Suganya and Selvanarayanan, 2010); *Erynia neoaphidis* (Remaudière et Hennebert) (Fuentes-Contreras *et al.* 1998); *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Poprawski *et al.*, 2000). Therefore, as part of the biopesticide development process, it is important to assess the compatibility of the candidate isolate with the crop

plant to be protected. This chapter reports on assessment of the effect of three host plant species on germination, virulence and persistence of two isolates of *M. anisopliae* against *M. vitrata* as part of the process to develop an effective biopesticide for managing the pest.

5.3 Materials and Methods

5.3.1 Plants

Three leguminous crops widely grown in Kenya and which are regular hosts of *M. vitrata* (Okeyo-Owuor *et al.,* 1983; Sharma *et al.,* 1999) were selected for this study. They included cowpea, *Vigna unguiculata* (L.) Walp variety "Ex-Lwanda", common bean, *Phaseolus vulgaris* L. variety "GLP2" (ROSECOCO) (Kenya Seed Co. Ltd), and pigeon pea, *Cajanus cajan* (L.) Millspaugh variety "Mbaazi-1" (Dryland Seed Ltd, Kenya), (Figure 5.1). Two groups of the three plants were used; the first group were planted in an open field, and provided flowers for the virulence and colony forming unit (CFU) bioassays. The second group of plants were planted in pots and maintained in a screen-house, and were used for the germination and fungal persistence bioassays.

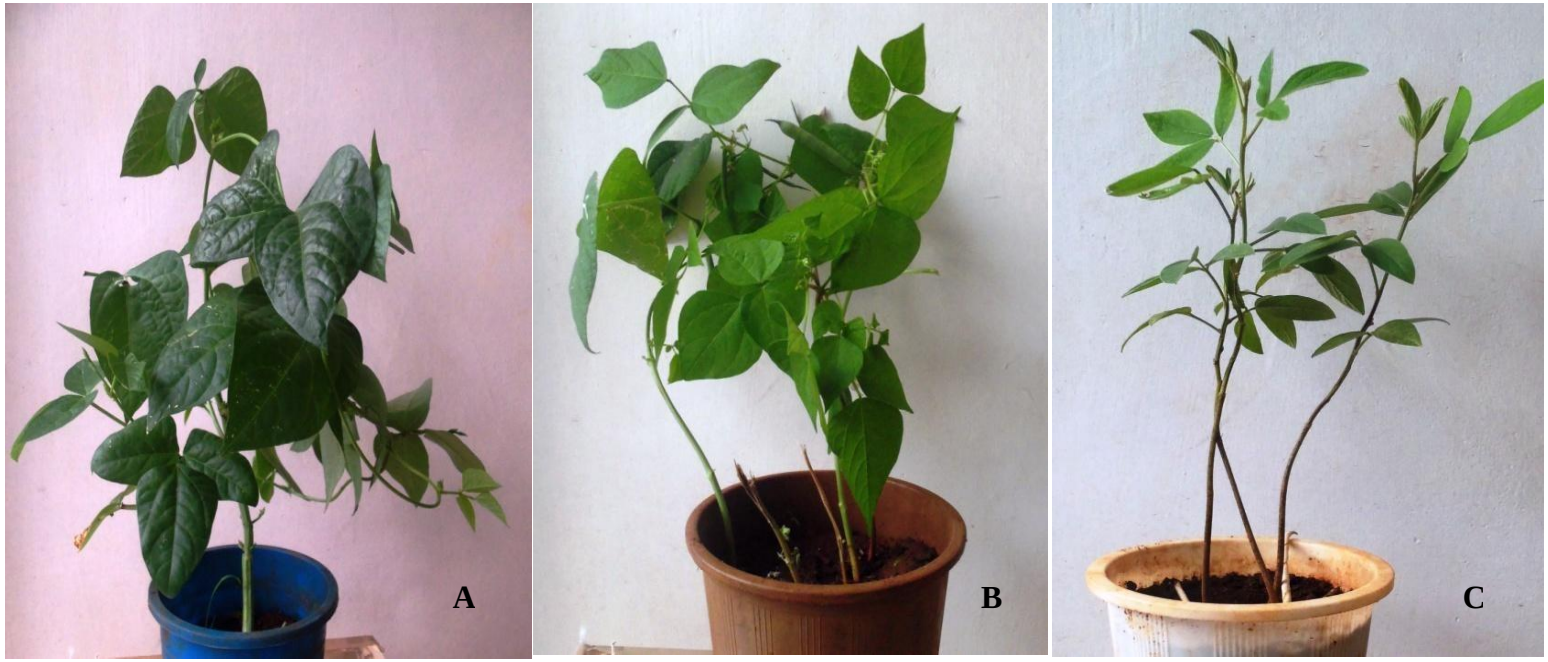


Figure 5.1 Host pants used in the study: (**A**) cowpea, *Vigna unguiculata*, (**B**) Common bean, *Phaseolus vulgaris* (**C**) Pigeon pea, *Cajanus cajan*. Photos: V. Tumuhaise.

5.3.2 Insects

Colony of *M. vitrata* originated from larvae obtained from naturally infested cowpea field located at *icipe*'s Thomas Odhiambo Centre located on the shores of Lake Victoria, Nyanza County, Kenya. The culture was maintained in the rearing room at 26 – 30ºC, 60 – 75% RH, with a 12 L:12 D photoperiod. Adults were fed on 10% sugar solution from balls of cotton wool soaked in the solution placed at the bottom corner of the rearing cages. The larvae were reared on semi-synthetic diet following the procedure described by Onyango and Ochieng'-Odero (1993) for over 15 generations before commencement of the experiments. The laboratory colony was rejuvenated with wild populations of *M. vitrata* every six months to maintain genetic vigour. Seedlings of the 3 test plants were provided to the adult females for oviposition. Seedlings carrying the eggs were harvested after 12 h and incubated for 3 days in the rearing room at 26 – 30ºC, 60 – 75% RH, for egg hatching. First instar larvae were then used in the virulence bioassay involving the respective plant species on which the eggs had been oviposited.

5.3.3 Fungi

Metarhizium anisopliae isolates ICIPE 18 and ICIPE 69 used in this study were obtained from the Microbial Germplasm of the *icipe*'s Arthropod Pathology Unit. They were selected as the most virulent isolates from the primary screening study involving 14 isolates of *M. anisopliae* and 6 of *B. bassiana* (Chapter Three). For both fungal isolates, conidial suspensions were prepared by harvesting conidia from 2 – 3 weeks old sporulating cultures grown on Saboraud dextrose agar (SDA) medium. The conidia were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in glass bottles containing 10 glass beads (3 mm). The suspension was vortexed on a mechanical shaker (5 min, 10 cm of vertical travel and 700 oscillations per min) to produce a homogeneous suspension. The conidia were then quantified following the procedure described by Inglis *et al.* (2012). Before undertaking any bioassay, conidial viability was assessed by spread-

plating 0.1 ml of a suspension of 3 x 10^6 conidia ml^{-1} on SDA medium. Inoculated plates were sealed with Parafilm membrane and incubated at 25 ± 2°C under complete darkness. At 24 h post inoculation, germination was terminated by adding a drop of 0.5% formaldehyde onto each plate. Two sterile microscope cover slips were then placed on each plate, and percentage germination determined by counting 100 spores under each cover slip on each Petri plate under the light microscope (x 400 magnification). A conidium was considered to have germinated if the germ-tube was at least twice the size of the spore (Goettel and Inglis, 1997; Inglis *et al.*, 2012). Each plate served as a replicate with four replications per test isolate. Cultures with ≥ 80% germination were used for bioassay.

5.3.4 Virulence of *Metarhizium anisopliae* against *Maruca vitrata* larvae maintained on different host plants

Fresh flowers of *V. unguiculata, C. cajan,* and *P. vulgaris* were arranged in Petri plates and sprayed with 10 ml of standard concentration of 1 × 10^8 conidia ml^{-1} fungal suspensions, using the Burgerjon spray tower (Burgerjon, 1956). Control lots consisted flowers of the three plants treated with sterile distilled water containing 0.05% Triton X100. The treated flowers were thereafter transferred into ventilated clear plastic dishes (11 x 4 cm). Twenty, first-instar larvae of *M. vitrata* were placed onto the treated flowers and left to feed for 24 h. Thereafter, insects were maintained on untreated fresh flowers which were added daily throughout the experimental period. The insects were monitored daily for 7 days for mortality. Dead insects were collected and transferred into Petri plates lined with moist filter paper to facilitate mycosis. Four replications were used per treatment.

5.3.5 Effect of host plant flowers on colony forming units

Five freshly harvested flowers of each host plant from field plots of *V. unguiculata*, *P. vulgaris*, and *C. cajanus*, respectively, were dipped for 30 sec. in 10 ml of 1 x 10^8 conidia ml^{-1} of *M. anisopliae* isolate ICIPE 18 or ICIPE 69 prepared as described above. One sterile filter paper was also dipped in either isolate to serve as a positive control. A negative control was also included and consisted of flowers of the 3 host plants dipped in sterile water containing 0.05% Triton X-100. The treated substrates were separately placed in sterile Petri plates lined with sterile filter paper to drain off excess suspension. The plates were then sealed with Parafilm membrane, and kept for 12 h at 25 ± 2°C. The flowers were thereafter suspended in 10 ml of sterile distilled water containing 0.05% Triton X100 in 25ml glass bottles with glass beads, and vortexed for 2 min to dislodge conidia from the flowers. The concentration of the resultant suspension was titrated to 1 x 10^3 conidia ml^{-1}, and 0.1 ml of which was spread-plated on selective media. The plates were sealed with Parafilm membrane, and incubated at 25 ± 2°C for 48 h, after which the number of colony forming units (CFU) per plate was assessed. Three replicate plates were used per treatment.

5.3.6 Effect of host plant leaf substrate on conidia germination

Leaves were freshly harvested and within 30 mins, two rectangular pieces (20 x 40 mm) of leaf were cut out from each of the three host plant species, and arranged at equal distance in a plastic Petri dish (90 mm). A piece of sterile filter paper (20 x 40 mm) was also included as a control. The substrates were then sprayed with 10 ml of 1 x 10^8 conidia ml^{-1} fungal suspension using the Burgerjon spray tower. Thereafter, they were separately transferred into sterile Petri dishes, sealed with Parafilm membrane and kept for 12 h at 25 ± 2°C. They were then suspended in 10 ml of sterile distilled water containing 0.05% Triton X100 in 25-ml glass bottles with 10 glass beads (3mm diameter), and vortexed for 2 min to dislodge the conidia. The resultant suspension was filtered through cheese cloth,

and 0.1 ml of the filtrate was spread-plated on Sabouraud dextrose agar + yeast (SDAY) amended with 500 µg/ml chloramphenicol. Spore germination was assessed after 24 h as above. Five replicate plates were used per treatment.

5.3.7 Persistence of *Metarhizium anisopliae* on different host plants

Inoculation of plants with fungus*:* Two-months old potted plants of *V. unguiculata*, *P. vulgaris*, and *C. cajanus* (Figure 5.1) raised in the screen house were transferred into cages (60 x 60 x 60 cm), with one plant per cage, and kept in the open environment. The cages had a wooden base and roof, with three sides of mosquito net fitting and the fourth side with a sliding Perspex door (Figure 5.2).

Figure 5.2 A representative experimental cage (60 x 60 x 60 cm) loaded with potted cowpea seedling. Photo: V. Tumuhaise

The wooden roof thus protected the plants from direct sunlight. At this stage, *V. unguiculata* and *P. vulgaris* had started flowering, while *C. cajanus* had not initiated flowering. The plants were sprayed until runoff, with 1 x 10^8 conidia ml^{-1} aqueous formulation of *M. anisopliae* isolate 1CIPE 18 or ICIPE 69. Plants in the control were sprayed with sterile distilled water containing 0.05% Triton X-100. Two plants were sprayed per treatment, and served as source of leaf substrates for CFU assessment.

Assessment of fungal persistence: On each sampling occasion, leaf samples were randomly harvested from the treated plants at 0, 3, 6, 9 and 14 days after treatment, placed separately in sterile Petri plates. Only leaves that were present at the time of spraying were harvested for CFU assessment, two leaf discs (each 5mm diameter were cut using a sterile cork borer, from one leaf of *V. unguiculata*, *P. vulgaris*, and *C. cajanus* treated with isolate ICIPE 18, ICIPE 69 or sterile Triton water. The leaf discs were then transferred according to treatments, into 25 ml universal bottles each containing 10 ml of 0.05% Triton water and 10 glass beads (3-mm diameter). Bottle contents were vortexed on a mechanical shaker (5 min, 10 cm of vertical travel and 700 oscillations per min) to dislodge fungal spores from the leaf discs. Presence of viable fungal spores was assessed by spread-plating 0.1 ml of the resultant suspension on selective media (Goettel and Inglis, 1997; Inglis *et al.*, 2012). Inoculated plates were sealed with Parafilm membrane and incubated at 25 ± 2°C in the dark for 4 days, after which the number of CFU was recorded. Five replicate plates were used per treatment.

5.3.8 Statistical analyses

All analyses were done using R v2.15.1 (R Development Core Team, 2008). From the virulence bioassay, larval mortality in the control lots for all host plants exceeded 5%, and mortality data were thus subjected to Abbott correction (Abbott, 1925), to correct for natural mortality before performing ANOVA to generate means. Mortality data recorded over the 7 days period were to generate LT_{50} estimates along with slopes of the regression

curves for each replication using Generalised Linear Model (GLM), which were then subjected to ANOVA to generate their means. Spore germination as percentage was subjected to square root transformation (Gomez and Gomez, 1984), and thereafter subjected to ANOVA to generate means. Data for the colony forming units (CFU) as affected by host plant flowers were subjected to ANOVA. On the other hand, CFU data for assessing fungal persistence for 14-days period was square-root transformed before performing the ANOVA. In all cases, whenever treatment effects from ANOVA were found to be significant (P = 0.05), means were separated using Tukey's HSD test (P = 0.05).

5.4 Results

5.4.1 Effect of host plant on the virulence of *Metarhizium anisopliae* against *Maruca vitrata*

Virulence of *M. anisopliae* isolate ICIPE 18 against *M. vitrata* larvae varied significantly across the three plants (F = 7.34; df = 2, 9; P = 0.0129). Larvae maintained on cowpea flowers suffered highest mortality rate compared to those on *P. vulgaris* and *C. cajan (*Table 5.1). Similarly, significant differences were recorded in isolate ICIPE 69 across the 3 host plants (F = 8.86; df = 2, 9; P = 0.0075). In contrast, highest mortality was recorded on *P. vulgaris* for this isolate (Table 5.1). Comparison of fungal isolates per plant revealed significant differences only on *P. vulgaris* (F = 36.51; df = 1, 6; P = 0.0009).

There were no significant differences among the LT_{50} estimates for isolate ICIPE 18 across the host plants (F = 0.74; df = 2, 9; P = 0.5025), and their respective slopes (F = 0.71; df = 2, 9; P = 0.5171). For ICIPE 69, significant differences were observed among LT_{50} estimates (F = 18.55; df = 2, 9; P = 0.0006), and slopes (F = 8.67; df = 2, 9; P = 0.008) for the host plants (Table 5.1).

5.4.2 Effect of host plant flower on colony forming units of two isolates of *Metarhizium anisopliae*

The number of colony forming units (CFU) for isolate ICIPE 69 was significantly affected by host plant (F = 6.60; df = 3, 8; P = 0.0148). Isolate ICIPE 69 conidia exposed flowers of *C. cajan* flowers yielded fewer CFU compared to those exposed to flowers of *V. unguiculata* and *P. vulgaris*, or the control (Table 5.2). On the other hand, no differences were noted in the number of CFU for isolate ICIPE 18 under the different host plants (F = 0.39; df = 3, 8; P = 0.7613). Differences in CFU due to fungal isolate were only observed for isolate ICIPE 69 on *P. vulgaris* (F = 148.26; df = 1, 4; P = 0.0003).

5.4.3 Effect of leaf substrate on germination of *Metarhizium anisopliae* isolates ICIPE 18 and CIPE 69

Both fungal isolates ICIPE 18 and 69 exposed to *V. unguiculata*, *C. cajan* and *P. vulgaris*, as well as control, had high spore germination rates in the range of 96.3 – 98.6% (Figure 5.2).

Table 5.1 Mortality and speed of kill of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 against first instar larva of *Maruca vitrata* as influenced by three host plants

	% mortality ± SE		LT_{50} ± SE		Slope ± SE	
Host plant	ICIPE 18	ICIPE 69	ICIPE 18	ICIPE 69	ICIPE 18	ICIPE 69
P. vulgaris	36.6 ± 9.4bB	95.7 ± 2.5aA	3.7 ± 1.3aA	0.7 ± 0.2bA	1.6 ± 0.7aA	3.9 ± 1.2bA
V. unguiculata	77.5 ± 3.2aA	76.2 ± 4.5abA	1.6 ± 0.6aA	1.3 ± 0.6bA	2.5 ± 0.7aA	1.9 ± 0.7bA
C. cajan	54.9 ± 8.5abA	57.0 ± 10.0bA	3.7 ± 1.9aA	5.8 ± 0.9aA	2.8 ± 0.8aB	7.1 ± 0.6aA

Means under each parameter followed by the same lower case letter within a column, and upper case letter within a row, are not significantly different by Tukey's HSD test ($P = 0.05$)

Table 5.2 Colony forming units (CFU) recorded in *M. anisopliae* isolates ICIPE 18 and ICIPE 69 exposed to flowers of 3 host plants

	Mean CFU ± SE	
Host plant	Isolate ICIPE 18	Isolate ICIPE 69
Control	28.0 ± 5.9Aa	25.0 ± 7.1Aab
P. vulgaris	29.7 ± 2.9Aa	29.0 ± 2.5Aa
V. unguiculata	26.0 ± 1.5Aa	27.3 ± 1.7Aa
C. cajan	31.0 ± 1.5Aa	7.3 ± 1.2Bb

Means followed by the same lower case letter within a column, and upper case letter within a row are not significantly different by Tukey's HSD test ($P = 0.05$)

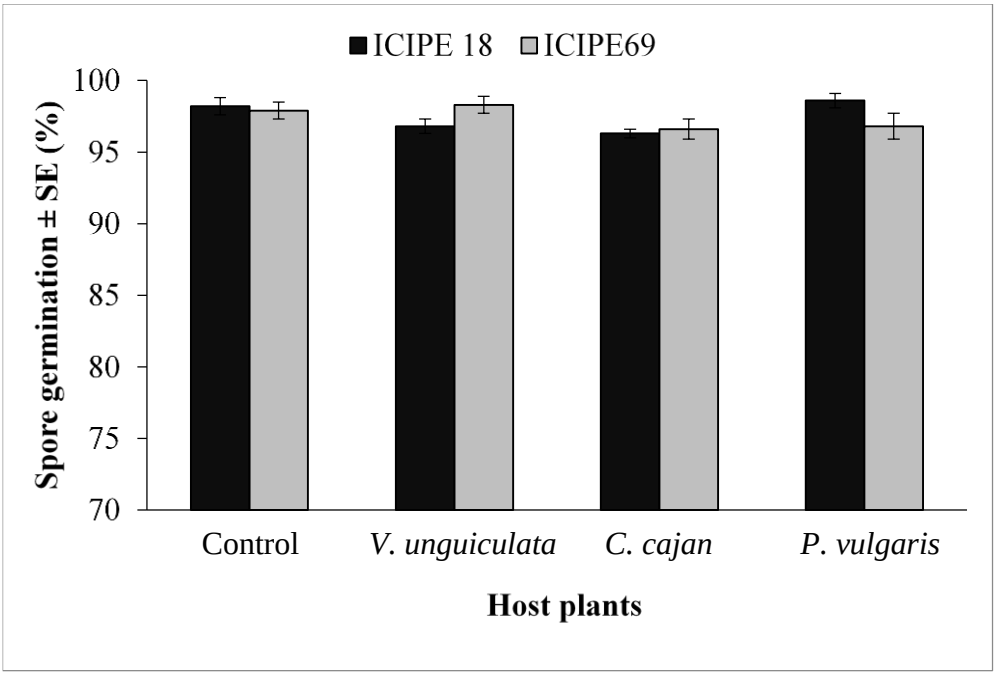


Figure 5.3 Germination of isolates ICIPE 18 and ICIPE 69 conidia exposed to leaves of different host plants for 12 h at 25 ± 2°C

5.4.4 Persistence of two isolates of *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 on *Vigna unguiculata, Phaseolus vulgaris* and *Cajanus cajan*

All treatment factors, i.e., fungal isolate, host plant and time, and their interactions had significant effects on the fungal persistence (Table 5.3). The number of CFU was negatively correlated with time across all the three host plants for both isolates (Table 5.4). There were significant differences in regression coefficient (R^2) for the different host plants (F = 14.48; df = 5, 24; P <0.0001). *Phaseolus vulgaris* had a significantly higher R^2 estimate for both isolates ICIPE 18 and ICIPE 69, compared with *V. unguiculata* and *C. cajan*. Slopes of regression lines also varied significantly (F = 41.09; df = 5, 24; P < 0.0001), with *C. cajan* recording the steepest slope for both isolate ICIPE 18 and ICIPE 69; implying that fungal conidia exposed to *C. cajan* lost viability at a significantly higher rate compared with those exposed to *P. vulgaris* and cowpea. All the slopes were significantly different from zero (P < 0.0001).

The number of CFU recorded immediately after spraying were significantly higher compared to the subsequent sampling days for both isolates, ICIPE 18 and ICIPE 69, and all the three host plants (Table 5.5). At day zero, *C. cajan* had significantly higher number of CFU than *P. vulgaris* and *V. unguiculata*, for both fungal isolates. By day 3, more than 90% reduction in CFU was recorded for both isolates across the different host plants. *Phaseolus vulgaris* had a relatively higher number of CFU by day 3 and 6 compared to *V. unguiculata* and *C. cajan*. Beyond day 6, negligible number of CFU was recorded for both isolates ICIPE 18 and ICIPE 69 across the three host plants (Table 5.5). *Cajanus cajan* had the steepest slope of the regression equation for CFU over time for both isolates ICIPE 18 and ICIPE 69 (Table 5.4), implying that it was associated with a higher rate of spore viability loss, compared with *V. unguiculata* and *P. vulgaris*.

Table 5.3 Analysis of variance for colony forming units due to *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 exposed to 3 host plants for 14 days

Factor/ Source	df	*F*	*P*
Fungus	2	163.29	<0.0001
Plant	2	99.21	<0.0001
Time	4	512.72	<0.0001
Fungus x Plant	4	29.03	<0.0001
Fungus x Time	8	133.55	<0.0001
Plant x Time	8	107.99	<0.0001
Fungus x Plant x Time	16	31.69	<0.0001

Table 5.4 Regression coefficients for CFU due to isolates of *Metarhizium anisopliae* exposed to 3 host plants for 14 days

Fungal isolate	Host plant	R^2 ± SE	Slope ± SE	$F_{(1,23)}$	*P*
ICIPE 18	*P. vulgaris*	0.75 ± 0.02a	-2.48 ± 0.13ab	62.8031	5.04×10^{-8}
	V. unguiculata	0.56 ± 0.01bc	-2.36 ± 0.10ab	28.2688	2.13×10^{-5}
	C. cajan	0.53 ± 0.01c	-4.05 ± 0.24c	25.3384	4.29×10^{-5}
ICIPE 69	*P. vulgaris*	0.72 ± 0.02a	-2.97 ± 0.12b	55.3192	1.46×10^{-7}
	V. unguiculata	0.65 ± 0.03ab	-2.18 ± 0.18a	36.0360	4.03×10^{-6}
	C. cajan	0.55 ± 0.04bc	-4.81 ± 0.18d	23.2895	7.18×10^{-5}

Means within a column followed by the same lower case letter are not significantly different by Tukey's HSD test ($P = 0.05$). Negative values for the slope imply the negative correlation between CFU and time.

Table 5.5 CFU (mm^{-2} leaf) for *Metarhizium anisopliae* isolates ICIPE 18 and ICIPE 69 exposed to 3 host plants over a 14-day period

		Day after emergence				
Fungal isolate	Plant	0	3	6	9	14
ICIPE 18	*P. vulgaris*	1727 ± 169b	261 ± 27a	248 ± 36a	13 ± 3b	12 ± 1a
	V. unguiculata	1941 ± 161b	77 ± 13b	50 ± 13b	32 ± 2a	8 ± 1a
	C. cajan	5449 ± 590a	131 ± 18b	44 ± 7b	37 ± 4a	7 ± 2a
ICIPE 69	*P. vulgaris*	2517 ± 188b	308 ± 30a	228 ± 41a	17 ± 2a	13 ± 2b
	V. unguiculata	1459 ± 260c	117 ± 22b	54 ± 11b	8 ± 2a	5 ± 1b
	C. cajan	7511 ± 531a	80 ± 6b	37 ± 12b	14 ± 4a	3 ± 0a

Means within a column followed by the same lower case letter under each fungal isolate, are not significantly different by Tukey's HSD test ($P = 0.05$)

5.5 Discussion

Maruca vitrata larval mortality varied between the fungal isolates, and across the three host plants, with isolate ICIPE 18 being less virulent on *P. vulgaris* (36.6 ± 9.4) and ICIPE 69 on *C. cajan* (57.0 ± 10.0). This result could be suggesting presence of antifungal properties in both *P. vulgaris* and *C. cajan* that selectively act against isolates ICIPE 18 and ICIPE 69, respectively. It has also been demonstrated that inter- and intraspecific variability and the genetic diversity exist in *Metarhizium* and other entomopathogenic fungi (Tiago *et al.*, 2014), which could be responsible for the observed differences between isolates ICIPE 18 and ICIPE 69 in this study. These results do concur with findings from other tritrophic interaction studies reported elsewhere (Poprawski *et al.*, 2000; Ekesi *et al.*, 2000a; Meekes, 2001; Suganya and Selvanarayanan, 2010). For example, Ekesi *et al.* (2000) reported differences in virulence of *M. anisopliae* isolate ICIPE 69 against adult *Megalurothrips sjostedti* on three cowpea varieties with varying levels of resistance against key pests. They found that variety ICV 7, tolerant to *M. sjostedti* and common diseases induced lowest *M. sjostedti* mortality, compared with variety ICV2, susceptible to *M. sjostedti* but with good tolerance to pod bugs and pod borers, and variety ICV8, moderately resistant to *M. sjostedti* and pod borers. They also noted that only the tolerant variety significantly reduced spore germination and colony forming units of ICIPE 69. These differences were attributed to existence of antifungal substances in the tolerant variety (Ekesi *et al.*, 2000a).

In the present study, the observed differences in virulence led to further investigation into possible effect of plant compounds on germination and colony forming unit in the *M. vitrata – M. anisopliae* interaction. Although no plant effects were observed in terms of germination of *M. anisopliae* spores exposed to the different plants, CFU varied between fungal isolates and across host plants varied significantly. Application of the spores on *C. cajan* induced 3 – 4 times fewer CFU for isolate ICIPE 69, compared to *P. vulgaris* and *V. unguiculata*. The fewer CFU of isolate ICIPE 69 exposed to *C. cajan* flowers

correspond with low mortality rate for the same treatment under the virulence bioassay, thus suggesting the presence of antifungal substances in *C. cajan*.

Plant effects in insect-pathogen interactions can be direct or indirect or both (Butt and Goettel, 2000; Cory and Ericsson, 2010; Vega *et al.*, 2012). Examples of direct effects may include plant exudates affecting the conidia directly, or herbivore-induced plant volatiles affecting sporulation or germination (Cory and Ericsson, 2010). On the other hand, indirect effects may include plant quality, either allelochemicals or nutrients, altering insect condition (e.g. immunity), and thus disease resistance. It could also be the nutritional quality altering insect morphology (e.g. cuticle depth), which in turn would influence the infection process (Cory and Ericsson, 2010). Moreover, previous studies have demonstrated presence of antifungal compounds in *C. cajan*, such as isoflavonoid phytoalexins – hydroxygenistein, genistein, cajanin and cajanol (Marley and Hillocks, 1993, 2002; Nix *et al.*, 2015). *Cajanus cajan* has also been found to possess phytochemicals active against fungi of human health importance (Pal *et al.*, 2011; Oke, 2014).

Persistence of fungal biopesticide in the habitat of the target insect pest is an important factor in achieving effective management of the pest. Results from this study revealed that the treatment factors namely host plant, fungal isolate, and time, singly or through their interaction, affected the persistence of both *M. anisopliae* isolates). At 0 days post treatment, more spores were recovered from *C. cajan* compared to *P. vulgaris* and *V. unguiculata* but by day 3, more than 90% reduction in CFU was recorded for both isolates across the different host plants. There are similarities and differences between the present study and previous studies. Thus, as this study recorded differences in CFU count between *V. unguiculata*, *P. vulgaris* and *C. cajan* at 0 days post treatment, Gatarayiha *et al.* (2010) also observed differences in *B. bassiana* CFU counts from leaves of beans, cucumber, eggplant, maize, and tomato collected immediately after *B. bassiana* spray. On the other

hand, no such differences were observed by Kouassi *et al.* (2003) in their study of *B. bassiana* persistence on lettuce and celery. Such decline in CFU counts over time is also reported by Ekesi *et al.* (2001); Kouassi *et al.* (2003) and Gatarayiha *et al.* (2010). For example, Ekesi *et al.* (2001) noted that *M. anisopliae* isolate ICIPE 69 remained active in the cowpea field for 3 – 4 days. The rate of decline of CFU counts are also reported to vary between host plants (Kouassi *et al.*, 2003; Gatarayiha *et al.*, 2010). The differences observed among the host plants in the present study are attributed to morphological and/or biochemical differences between plants. Morphological features may include waxes, trichomes and lattices. Indeed, variability in type and density of trichomes has been reported in varieties of *P. vulgaris* (Dahlin *et al.*, 1992; Park *et al.*, 1994), *C. cajan*, and wild *Cajanus* spp. (Romeis *et al.*, 1999; Sunitha *et al.*, 2008), and *V. unguiculata* (Jackai and Oghiakhe, 1989; Oghiakhe *et al.*, 1992). Additionally, some types of trichomes (i.e. glandular secreting trichomes) are reported to exude phytochemicals such as terpenes, phenolics, alkaloids or other substances, which complement the plant's chemical defense against pests and pathogens (Wagner, 1991; Wagner *et al.*, 2004).

When fungal spores stay longer on the leaf surface, biochemical factor become more relevant as the spores are allowed more time to exert their effect on the fungal spores. This perhaps explains the observed rapid loss of conidia viability resulting in less than 10% CFU recorded 3 days after treatment, relative to the number recorded immediately after spray). This situation coud possibly be mitigated by manipulating the formulation of the fungal conidia especially using oil which is known to enhance efficacy of entomopathogens, compared to aqueous formulations (Brooks *et al.*, 2004; Wraight *et al.*, 2007; Jackson *et al.*, 2009; Jaronski, 2010). The steeper slope associated with *C. cajan* suggests a relatively higher rate of viability loss on this plant, compared with *V. unguiculata* or *P. vulgaris*. This observation again, coincides with the observed low virulence and fewer CFU of *M. anisopliae* ICIPE 69 exposed to the same host plant (*C. cajan*), further strongly suggesting presence of antifungal substances. Additional studies

are needed on the nature of these compounds to guide implementation of biopesticide-based management options for *M. vitrata* on the target crops.

5.6 Conclusion

The present study has demonstrates that exposing the conidia of *M. anisopliae* isolates ICIPE 18 and ICIPE 69 to flowers of different host plants affects CFU and virulence against *M. vitrata*. On the contrary, germination is not affected by exposing the fungal conidia to intact leaves of *V. unguiculata*, *P. vulgaris*, and *C. cajan*. The differences in CFU, virulence, and persistence suggest presence of antifungal properties in the test plants especially *C. cajan*. The study also demonstrates that aqueous formulated *M. anisopliae* can hardly persist beyond 3 days post spraying. Overall, the results suggest that host plant can significantly alter the efficacy of fungal-based biopesticide, and that interaction between the target host plant and the fungal isolate should routinely be investigated as part of the biopesticide development process.

CHAPTER SIX

FIELD EVALUATION OF *METARHIZIUM ANISOPLIAE*-BASED COMMERCIAL BIOPESTICIDE (CAMPAIGN®) AND NEEM (NIMBECIDINE®) FOR THE MANAGEMENT OF *MARUCA VITRATA* AND OTHER LEGUME PESTS ON COWPEA

6.1 Summary

Cowpea, an important food legume across the semi-arid tropics, is considered too risky an investment by most farmers because of numerous pests associated with it, and of which *Maruca vitrata* and thrips being regarded as among the most damaging species. Use of chemical pesticides is the most widely known form of pest control on cowpea. In an effort to identify potent isolates against *Maruca vitrata*, laboratory studies identified *M anisopliae* isolates ICIPE 18 and ICIPE 69 that werevirulent against *M. vitrata*. Additionally, ICIPE 69 had better mass production properties than ICIPE 69. On the basis of these results, commercial formulation of *M. anisopliae* ICIPE 69, Campaign® was field-tested for the management of *M. vitrata* on cowpea in Coastal region, Kenya during the 2012 and 2013 cropping seasons. This mycopesticide was compared with Nimbecidine® (neem) and Karate® (Lambda-cyhalothrin). Karate®, Campaign® and Nimbecidine®, significantly reduced damage by *M. vitrata*, resulting into yield increment over the control of up to 1254 Kg/ha (387%), 747kgs/ha (231%), and 340 kg/ha (117%), respectively. This translated into marginal returns of up to 5.7 (Karate®), 3.1 (Campaign®) and 1.2 (Nimbecidine®). Considering the dangers associated with synthetic pesticides, the mycopesticide, Campaign® is therefore recommended as an effective protection tool for cowpea against insect pests.

6.2 Introduction

Cowpea is one of the most important food legumes in the semi-arid tropics covering Asia, Africa, southern Europe, and Central and South America (Singh, 2005; Timko and Singh, 2008). It is a multifunctional crop, providing food for humans and livestock; and a source of direct revenue for growers and income for traders of grains (Langyintuo *et al.*, 2003). Cowpea is a drought tolerant and warm weather crop, and as such, it is well adapted to the drier regions of the tropics, where other food legumes do not perform well (Singh, 2005). It is however considered a risky crop for investment by most farmers because of numerous pests associated with it, most of them appearing concurrently or overlapping across different phenological stages (Adati *et al.*, 2008; Timko and Singh, 2008; Srinivasan, 2014). Insect species which occur during the flowering and podding stages of the plant are the most damaging and among these, the legume pod borer, *Maruca vitrata* (Fabricius) (Lepidoptera: Crambidae) ranks very high (Sharma, 1998; Timko and Singh, 2008; Ganapathy, 2010; Srinivasan *et al.*, 2012).

Damage by *M. vitrata* is caused by the larval stage (Sharma, 1998; Sharma *et al.*, 1999; Ganapathy, 2010; Srinivasan, 2014). The larvae emerge from eggs in the early evening and wander on plant surfaces feeding on tender plant stems, terminal shoots, and peduncles during the vegetative growth, and on flowers as plants mature (Sharma, 1998; Ganapathy, 2010). The older larvae often bore into pods, and occasionally into peduncles and stems (Sharma, 1998; Ganapathy, 2010). The pest can cause up to 100% yield loss if not effectively controlled (Dugje *et al.*, 2009). Previous chapters have been able to demonstrate laboratory efficacy of *Metarhizium anisopliae* isolate ICIPE 18 and 69 in the management of *M. vitrata*. Among the two isolates, ICIPE 69 is highly amenable to mass production and has recently been commercialized as Campaign® for the management of selected insects pests (thrips and mealybug) of agricultural importance (www.icipe.org; www.realipm.com). Despite the potency of isolate ICIPE 69 as demonstrated against *M. vitrata* under laboratory conditions presented in the previous chapters, there are still no

documented facts on efficacy of this isolate against this pest in the field. This study was thus undertaken to evaluate the efficacy of *M. anisopliae* isolate ICIPE 69 by field-testing the commercial product Campaign® with the view of refining the recommendation of the product to include *M. vitrata* among the target pests.

Although, the target pest in this study was *M. vitrata*, field observations have shown rising importance of the black cowpea aphid, *Aphis craccivora* (Koch) (Homoptera: Aphididae) in many farms. In addition to its widespread occurrence in much of tropical Africa, *A. craccivora* also attacks cowpea in India, the Philippines, Thailand, and the southern United States (Jackai and Adalla, 1997; Adati *et al.*, 2008; Srinivasan, 2014). They feed on stems, terminal shoots, and petioles of seedlings and, as plants mature, they move to pods and flowers. Heavy feeding causes stunting of plants and delay in the initiation of flowering (Jackai and Adalla, 1997; Srinivasan, 2014). An indirect and generally more harmful effect of aphid, even of small populations, is the transmission of cowpea aphid-borne mosaic virus which severely reduces yield (Singh and Allen, 1979; Singh and Emden, 1979). In view of the above, the impact of the biopesticide on this aphid damage to cowpea was also assessed. Similarly, records were also kept of the efficacy of the product on thrips, specifically *Megalurothrips sjostedti* damage. Field application of neem has also been observed among smallholder cowpea growers for the management of cowpea pests, and in this regard Nimbecidine®, a neem-based biopesticide was included in the study to assess its relative performance in protecting the crop against pest damage.

6.3 Materials and methods

6.3.1 Experimental design and treatments

Field experiments were conducted at a farmer's field in Kilifi, Coastal region, Kenya during the short rains of 2012 (October – December) and 2013 (August – November). Cowpea variety ICV 2 was planted in 5 x 5 m plots with an intra-row spacing of 30 cm

and inter-row spacing of 60 cm. A distance of 5 m was maintained between plots and blocks. Variety ICV 2 matures in about 70 days, and is characterized by spreading indeterminate growth habit. Two weeks after germination, seedlings were thinned to one plant per stand and gap filling done to replace dead seedlings. Weeding was done as and when necessary to ensure clean plots. Experimental fields were laid out in randomized complete block design with each treatment replicated four times. The experiment had four treatments formulated as per manufacturer instructions as follows:

i) *Metarhizium anisopliae*-based biopesticide isolate ICIPE 69, registered in Kenya as Campaign® (Real IPM Company, Thika, Kenya) applied at the rate of 200 ml/ha of the commercial formulation containing 1 x 10^{11} colony forming units ml^{-1},

ii) Karate® (Lambda cyhalothrin), as a positive check, applied at the recommended rate of 1 litre ha^{-1} of the commercial formulation containing 17.5 g a.i. ha^{-1},

iii) Nimbecidine® at 2.2 litres ha^{-1} containing 2,565 ppm a.i. ha^{-1}, and

iv) Untreated control.

Application of treatments to the plants was done starting at 41 days after emergence (DAE), and repeated after 5 days. Spraying was done in the evening between 1700 h and 1830 h to protect *M. anisopliae* conidia, the active ingredient of Campaign® against the adverse effects of UV radiation (Zimmermann, 2007; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012). A separate sprayer was consistently used for each treatment to avoid contamination.

6.3.2 Damage and grain yield assessment

Data were collected on damage caused by three key pests namely *M. vitrata*, thrips and aphids (Figure 6.1) starting at 30 days after crop emergence (DAE), while grain yield data was recorded after harvesting (i.e. 65 DAE), following the procedures described below:

i) *Maruca vitrata*: Damage was assessed starting at 30 DAE following the procedure described by Afun *et al.* (1991). Twenty 20 flowers were collected at random from each treatment plot. They were carefully opened and thoroughly inspected for presence of *M. vitrata* larvae and/or damage/frass. The number of damaged flowers were then counted and expressed as percentage of the total flowers sampled (Afun *et al.*, 1991). Flowers damage was used as opposed to direct insect count due to problems associated with limited number of larvae that are frequently encountered on flowers as they voraciously move from flower to flower, and also most often carry out cannibalistic behavior. Damage data were also used to assess product efficacy for the other pests below.

ii) Thrips: Damage assessment commenced at 30 DAE following the procedure described by Jackai and Singh (1988), and Egho (2011) which involves visual estimation of browning and drying of terminal and floral buds. Twenty terminal or floral buds and/or flowers were randomly collected from each treatment plot, and thrips damage rated on a scale of 1 – 9 (Jackai and Singh, 1988; Egho, 2011) as follows: 1 = No browning/drying of stipules, leaf buds or flower buds; no bud abscission; 3 = Initiation of drying of stipules, leaf buds and flower buds but no flower bud abscission; 5 = Distinct browning/drying of stipules, leaf buds and flower buds; start of flower bud abscission; 7 = Serious flower bud abscission and failure of peduncles to elongate; 9 = Very severe bud abscission, and most plants carry short barren peduncles.

iii) Aphid: Damage was assessed starting at 30 DAE by selecting 20 plants randomly from the 2 middle rows of each treatment plot and examining them for the presence of aphids/aphid colonies. The size of aphid colony on each of the 20 plants was rated on a 0 – 9 point scale (Litsinger *et al.*, 1977; Egho, 2011) as follows: 0 = No infestation; 1 = Few individual aphids; 3 = Few small scattered colonies; 5 = Several small scattered colonies; 7 = Scattered pockets of large colonies; 9 = Large continuous colonies.

iv) Grain yield: This was estimated at 65 DAE by harvesting grain along the entire length of each row in each plot when the grains were dry. The pods were hand-picked, threshed, winnowed and then weighed. The results were extrapolated to kilogram per hectare.

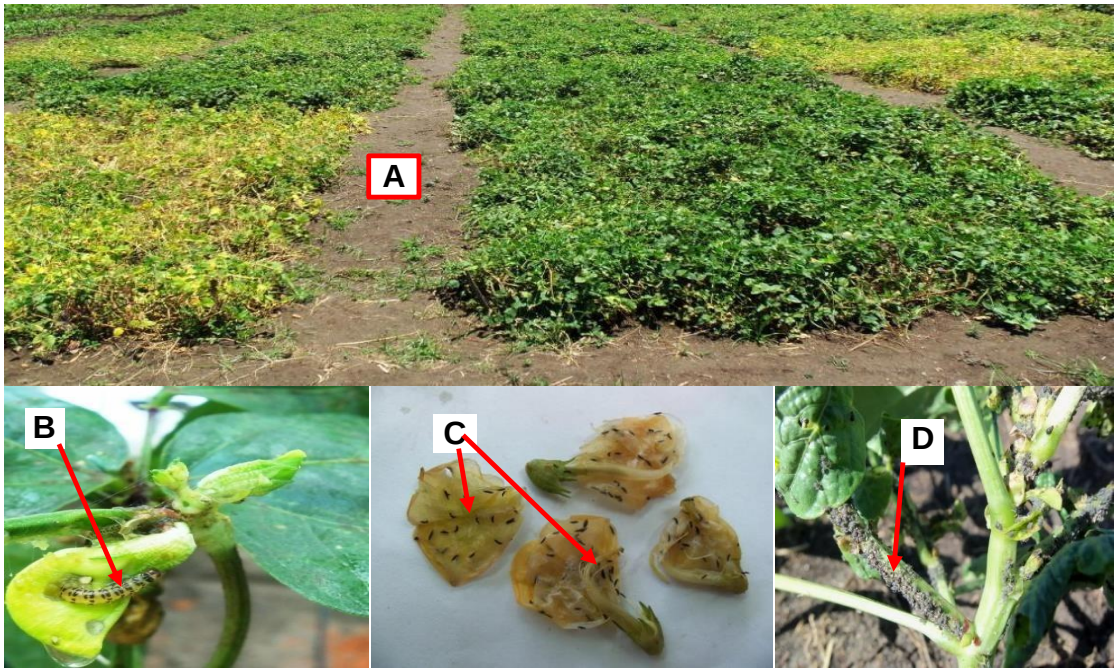


Figure 6.1 A section of cowpea field trial showing treatment effects across experimental plots (A); and the insect pests sampled; B: *Maruca vitrata* (coming out of the flower), C: thrips (appearing as black spots on the flowers), and D: aphids (on the leaf petiole). Photos: V. Tumuhaise.

6.3.3 Statistical analyses

Data analyses were done using R (R Development Core Team, 2008). Percent data for *M. vitrata* damage was arc-sine transformed and then subjected to ANOVA to generate means. On the other hand, thrips damage and aphid colony scores, and yield data were square-root transformed before performing ANOVA. Whenever treatment effects were

significant ($P = 0.05$), means were separated by Tukey's HSD. Relative effectiveness of the test pesticides was assessed by computing the decrease in pest damage by each treatment over the untreated control.

Economic benefit associated with the different pesticides was assessed by establishing the marginal returns following the method described by Karungi *et al.* (2000) and Nabirye *et al.* (2003). The costs associated with the different pesticide treatments are presented in Table 6.1, and were used to calculate the profitability (marginal returns) for each treatment. The marginal returns indicate the value of the yield gained due to a given treatment relative to the cost of that treatment. A value of marginal returns less than 1 implies that the increase in cowpea yield does not compensate for the cost of the treatment applied.

Table 6.1 Costs of pesticide application per season, involving two treatment sprays, used in calculating marginal returns

Treatment	Item	Cost (KES)[1]	Cost (USD)
Karate®	Pesticide for 4 sprayings[a]	8,000	94
	Knapsack sprayer[b]	3,360	40
	Labour for 4 sprayings[c]	2,000	24
	Additional labour to handle extra grain[c]	2,000	24
	Total	15,360	181
Campaign®	Biospesticide for 4 sprayings[a]	10,000	118
	Knapsack sprayer[b]	3,360	40
	Labour for 4 sprayings[c]	2,000	24
	Additional labour to handle extra grain[c]	1,500	18
	Total	16,860	198
Nimbecidine®	Biospesticide for 4 sprayings[a]	14,000	165
	Knapsack sprayer[b]	3,360	40
	Labour for 4 sprayings[c]	2,000	24
	Additional labour to handle extra grain[c]	1,000	12
	Total	20,360	240

Free market price at the time of the experiment; 1USD = KES 85

[a]Pesticide cost calculated/ha.

[b]Cost of sprayer and discounted over 5 years.

[c]Labour was calculated at 1 man-day; for harvesting, labour varies due to different quantities of produced by the various treatments.

6.4 Results

6.4.1 Damage caused by insect pests on cowpea

Maruca: Damage by *M. vitrata* was observed at 30 DAE across treatment at the time sampling was initiated (Figure 6.2) and ranged from 4.0 – 12.5% in 2012 and 16.3 – 62.5% in 2013. During this period, no significant differences were observed in damage across the various treatments. Treatment effects were noticed four days after spraying (i.e. 45 DAE) with damage levels significantly declining in the treated plots compared with the control plots, in both 2012 season ($F = 10.26$; df = 3, 12; $P = 0.0012$) and 2013 season ($F = 28.34$; df = 3, 12; $P < 0.0001$). Treatments had similar trends in both years, with Karate® being the most effective treatment while Campaign® was the second best.

Thrips: During the 2012 cropping season, treatment effects were noticed immediately after treatment application ($F = 9.18$; df = 3, 12; $P = 0.0020$). Damage was consistently higher in the untreated control plots and lowest in the Karate® treated plots. No significant differences were observed between Campaign® and Nimbecidine®. Similarly, treatment effects were noticeable immediately after spraying ($F = 4.47$; df = 3, 12; $P = 0.0251$). Campaign® was relatively more effective than Nimbecidine®, though not significantly different, while Karate® remained the most effective (Figure 6.3).

Aphids: Aphid infestation was generally low in the 2012 season (score: 0 – 1.75) from the first sampling date (30 DAE) up to 40 DAE with no significant differences across the experimental plots (Figure 6.4). However, 4 days after treatment application (45 DAE), treatment effects were observed ($F = 4.47$; df = 3, 12; $P = 0.0251$) with upsurge in aphid population (score: 4.0 – 4.5) recorded in plots treated with Campaign®, Nimbecidine® and the untreated control (Figure 6.4). Karate® recorded a knock-down effect and maintained the aphid population close to nil throughout the experimental period. Nimbecidine® also

caused a drop in aphid population after 45 DAE while the infestation remained unchecked in the untreated control plots and those treated with Campaign®.

During the year 2013, aphid infestation was higher at the onset of sampling (score: 4.0 – 6.5), as compared to the year 2012 (score: 0 – 1.0) (Figure 6.4). Significant differences were observed in the experimental plots prior to treatment application, at 40 DAE (F = 3.14; df = 3, 12; P = 0.0531), and populations continued to build up in the untreated control and the Campaign®-treated plots. On the other hand, significant treatment effects were recorded at 45 DAE (F = 13.70; df = 3, 12; P = 0.0004) with Karate® being the most effective treatment and Nimbecidine® ranking as second best.

Over all, the positive check Karate® was the most effective against all the three pests with 94.6 – 95.2%, 80.0 – 90.6% and 100% reduction in damage due to *M. vitrata*, thrips and aphids, respectively (Tables 6.2, 6.3, and 6.4). Campaign® was effective against *M. vitrata* and thrips achieving damage decrease of 67.6 – 82.5% and 70.6 – 73.3%, respectively but was not effective against aphids. On the other hand, Nimbecidine® was more effective against aphids achieving 73.3 – 78.6% damage reduction, and not effective against *M. vitrata* and thrips.

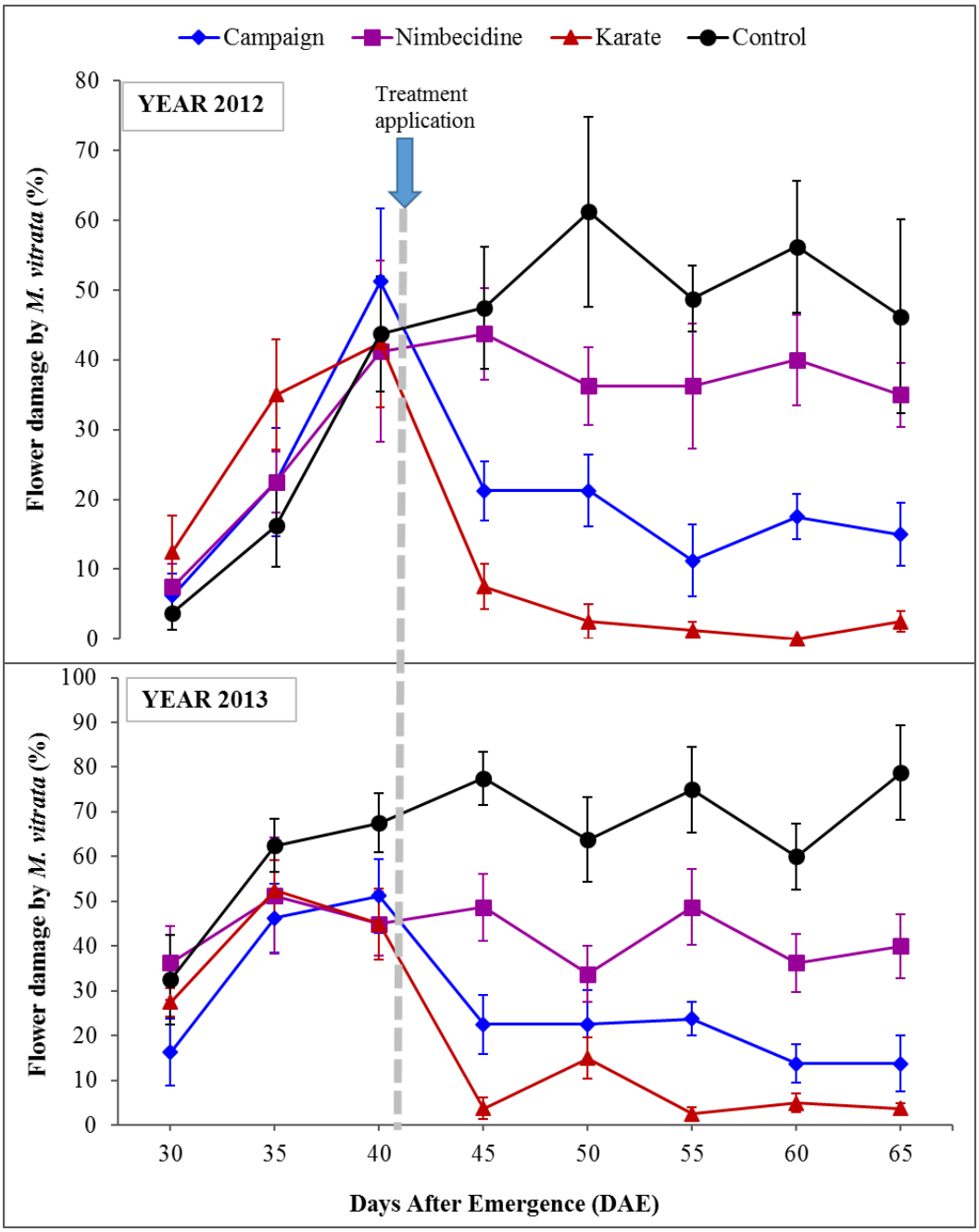


Figure 6.2 *Maruca vitrata* damage on cowpea during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya. Different letters at a given sampling day (DAE) imply significant differences by Tukey's HSD (P = 0.05).

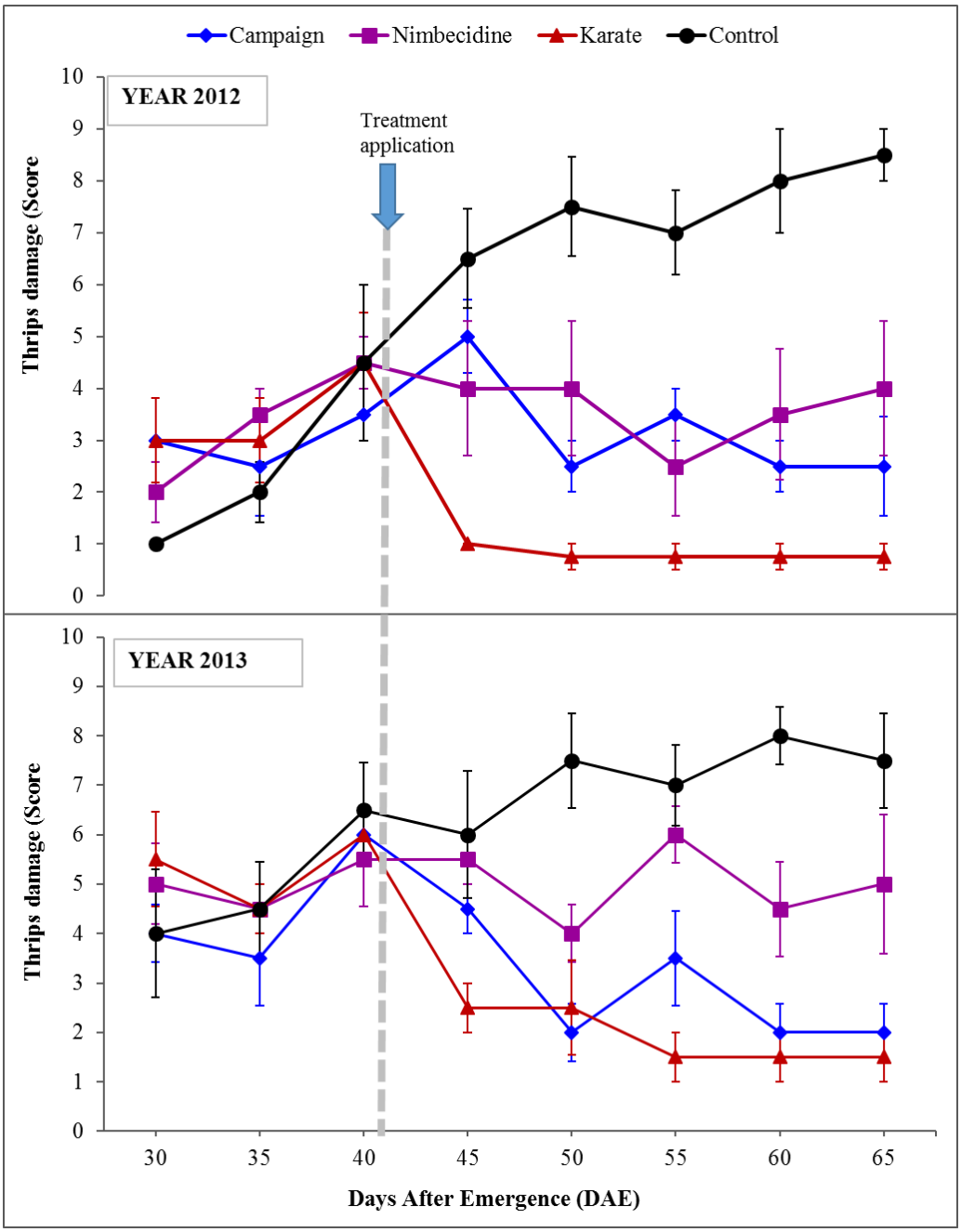


Figure 6.3 Thrips damage on cowpea during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya. Different letters at a given sampling day (DAE) imply significant differences by Tukey's HSD (P = 0.05).

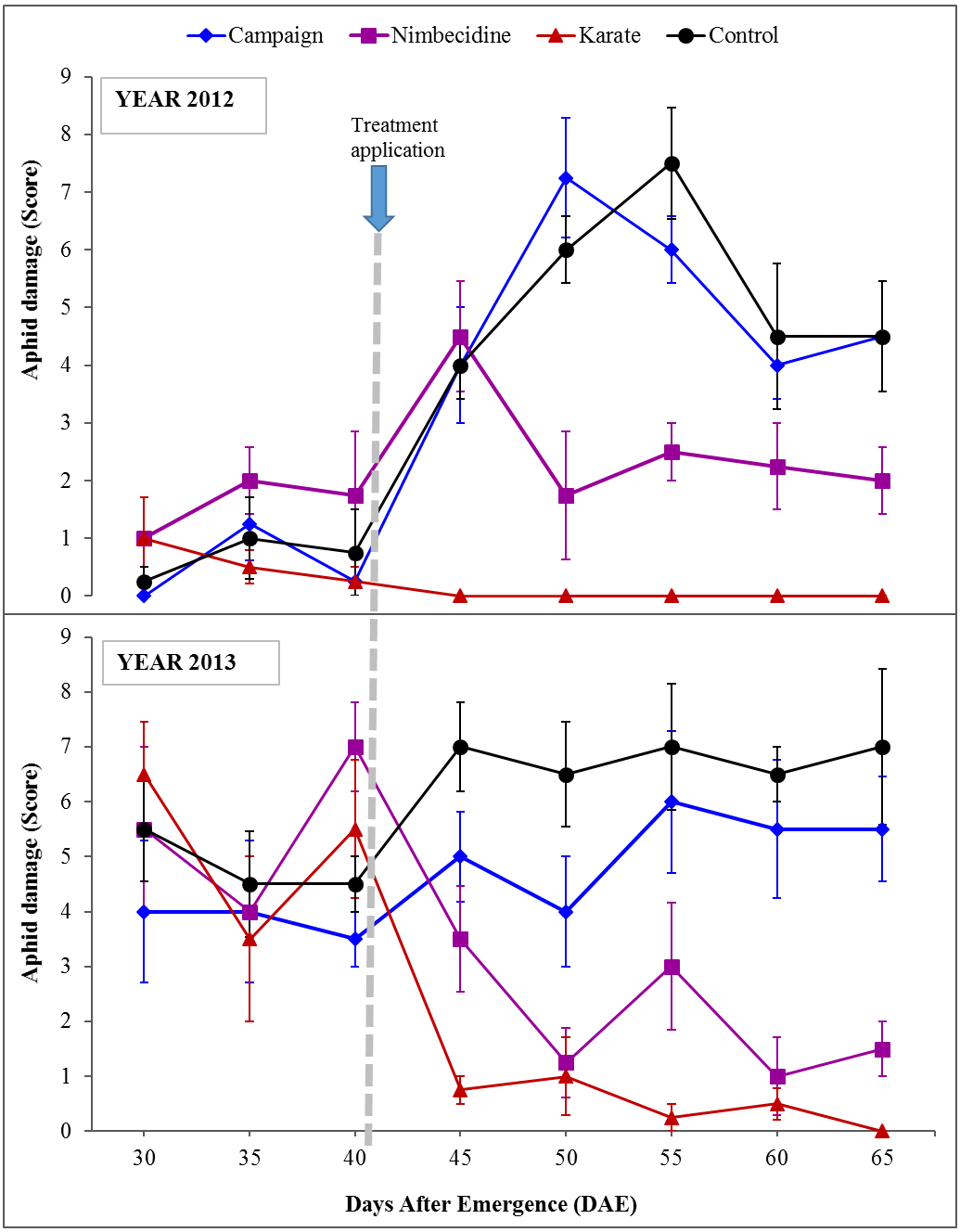


Figure 6.4 Aphid damage on cowpea during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya. Different letters at a given sampling day (DAE) imply significant differences by Tukey's HSD (P = 0.05).

Table 6.2 Effectiveness of Campaign®, Nimbecidine® and Karate® in reducing damage caused by *M. vitrata* on cowpea at 24 days after treatment (= 65 DAE) during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya

	2012		2013	
Treatment	% flower damage by *M. vitrata* (± SE)	% decrease over control	% flower damage by *M. vitrata* (± SE)	% decrease over control
Campaign®	15.0 ± 4.6ab	67.6	13.8 ± 6.3bc	82.5
Nimbecidine®	35.0 ± 4.6a	24.3	40.0 ± 7.1b	49.2
Karate®	2.5 ± 1.4b	94.6	3.8 ± 1.3c	95.2
Control	46.3 ± 13.9a	-	78.8 ± 10.5a	-

Means in the same column followed by the same letter do not differ significantly different by Tukey's HSD test ($P = 0.05$).

Table 6.3 Effectiveness of Campaign®, Nimbecidine® and Karate® in reducing damage caused by thrips on cowpea at 24 days after treatment (= 65 DAE) during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya

	2012		2013	
Treatment	Thrips damage (mean score ± SE)	% decrease over control	Thrips damage (mean score ± SE	% decrease over control
Campaign®	2.5 ± 1.0b	70.6	2.0 ± 0.6b	73.3
Nimbecidine®	4.0 ± 1.3b	52.9	5.0 ± 1.4ab	33.3
Karate®	0.8 ± 0.3b	90.6	1.5 ± 0.5b	80.0
Control	8.5 ± 0.5a	-	7.5 ± 1.0a	-

Means in the same column followed by the same letter do not differ significantly different by Tukey's HSD test ($P = 0.05$).

Table 6.4 Comparative effectiveness of Campaign®, Nimbecidine® and Karate® in reducing damage caused by aphids on cowpea at 24 days after treatment (= 65 DAE) during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya

	2012		2013	
Treatment	Aphid damage (mean score ± SE)	% decrease over control	Aphid damage (mean score ± SE)	% decrease over control
Campaign®	4.5 ± 1.0a	0.0	5.5 ± 1.0a	21.4
Nimbecidine®	2.0 ± 0.6a	55.6	1.5 ± 0.5b	78.6
Karate®	0.0b	100.0	0.0b	100.0
Control	4.5 ± 1.0a	-	7.0 ± 1.4a	-

For each species, means in the same column followed by the same letter do not differ significantly different by Tukey's HSD test ($P = 0.05$).

6.4.2 Cowpea grain yield and marginal returns

Treatment effects were consistent during both cropping seasons, and significantly different during each cropping season: 2012 season ($F = 36.85$; df = 3, 12; $P < 0.0001$) and 2013 season ($F = 28.88$; df = 3, 12; $P < 0.0001$). In both seasons, the positive check, Karate® was most effective in increasing grain yield followed by Campaign®.

The profitability analysis (marginal returns) also reveals Karate® to have the highest return on investment, with every dollar invested in protecting the cowpea crop yielding a net return of USD 4.4 – 5.7. Campaign® which was second best in increasing yield still ranked second in net monetary returns with each dollar invested yielding USD 2.7 – 3.1, while Nimbecidine® had the lowest returns of USD 1.1 – 1.2 (Table 6.5).

Table 6.5 Increment in grain yield and economic value obtained from protecting cowpea against insect pests using Campaign®, Nimbecidine® and Karate® during the 2012 and 2013 cropping seasons at Kilifi, Coastal Region, Kenya

Season and Treatment	Grain yield (kg/ha)	Yield increment over control (kg/ha)	% yield increment over control	Returns over control (USD/ha)[b]	Protection cost (USD/ha)[c]	Marginal returns[d]
Year: 2012						
Campaign®	1071.2 ± 52.6b	747.4	231	612.9	198	3.1
Nimbecidine®	664.4 ± 21.5c	340.6	105	279.3	240	1.2
Karate®	1578.2 ± 205.2a	1254.4	387	1028.6	181	5.7
Control	323.8 ± 31.1d	-		-	-	
Year: 2013						
Campaign®	905.5 ± 67.9ab	639.4	240	524.3	198	2.7
Nimbecidine®	576.6 ± 71.1b	310.5	117	254.6	240	1.1
Karate®	1233.4 ± 155.1a	967.3	364	793.2	181	4.4
Control	266.1 ± 19.9c	-		-	-	-

[b]Open market retail price for cowpea grain at the time of the study was KES 70 kg^{-1} [c]Cost of pesticide application derived from Table 6.1; [d]Marginal returns > 1 imply profitable investment**;** Forex exchange rate: 1 USD = KES 85.00

6.5 Discussion

Results from the two cropping seasons showed that application of the various pesticides had a significant impact on damage caused by the pest but this varied with the treatments. Karate® generally outperformed the other product in terms of efficacy on the three target pests. On the other hand, Campaign® was effective against *M. vitrata* and thrips while Nimbecidine® proved to significantly reduce damage by aphids but was less effective against *M. vitrata* and thrips. Damage in the untreated control plots was highest for all the three insect species throughout the two cropping seasons. Grain yield trends also followed the pattern observed for pest damage. Karate® which was effective against all the three pest species (*Maruca*, thrips, aphid) recorded the highest yield while Campaign®, ranked second in yield for its high level of efficacy against *M. vitrata*, thrips. Yield in the Nimbecidine® treatment ranked third but was significantly higher than the untreated control.

Maruca vitrata is generally regarded as a difficult pest to control due to the cryptic behaviour of the pest. However in these trials, this study has been able to demonstrate the efficacy of a biopesticide product based on *M. anisopliae* ICIPE 69 for the management of pest. Perhaps the behaviour of the pest in particular the nocturnal wandering of the larvae, (Sharma, 1998; Ganapathy, 2010) assures secondary uptake of spores from plant surface (tender plant stems, terminal shoots, peduncles and flowers). When the larvae return to shelter during the hot period of the day, microclimatic humidity in conceal plants structures ensure rapid conidia germination and impact on the pest (Inglis *et al.*, 2001; Cory and Ericsson, 2010; Vega *et al.*, 2012). It is probable that high relative humidity prevailing inside the flower buds, flowers, pods where *M. vitrata* inhabits facilitates germination of, and infection due to the fungal conidia picked by the insect and further underlining the contribution of microhabitat environment in promoting fungal infection. The fact that the biopesticide was applied in the evening also ensured that conidia were protected from the vagaries of ultraviolet radiation, and also maximized chances of early

larval instars of *M. vitrata* getting into contact with the fungal inoculums as they wandered over the plants through the night. This also demonstrates the importance of timing of application to assure maximum impact on the target pest. It is also important to note that Campaign® is formulated in oil, and the latter generally enhances efficacy of EPF (Inglis *et al.*, 2000; Inyang *et al.*, 2000; Jackson *et al.*, 2009; Kirubakaran *et al.*, 2013).

The present study also demonstrated that Campaign® is effective against thrips. This corroborates earlier studies in which non-commercial formulations of the active ingredient, *M. anisopliae* isolate ICIPE 69 was found effective against different species of legume thrips including the legume flower thrips, *M. sjostedti* (Ekesi *et al.*, 1998), and the western flower thrips, *Frankliniella occidentalis* (Pergande) (Niassy *et al.*, 2012a).

The reason for the inefficiency of Campaign® against the aphid, *A. cracivora* in the present study is unknown, and yet aphids are generally considered as one of the most vulnerable insect to epizootics of fungal diseases (Milner, 1997). Moreover, previous studies have reported efficacy of EPF including *M. anisolpliae*, against the cowpea aphid, *Aphis craccivora* (Ekesi *et al.*, 2000b; Nirmala *et al.*, 2006; Saranya *et al.*, 2010; Suresh *et al.*, 2012). For example Saranya *et al.* (2010) reported up to 80.76% aphid mortality due to *M. anisopliae* under laboratory conditions.

It is known that EPF are generally host specific, and as such isolates of EPF vary in their pathogenicity to different insects (Sandhu *et al.*, 2012; Vega *et al.*, 2012). For example, Dimbi *et al.* (2003) found one *M. anisopliae* isolates ICIPE 60 that was among the isolates effective against one African Fruit Fly species *C. rosa* var. *fasciventris* Karsch., causing 89.7% mortality was yet found ineffective against the other fruit fly species, *Ceratitis capitata* (Wiedemann), causing only 34.7% mortality. From the same study, there were other *M. anisopliae* isolates, including isolate ICIPE 69 active against *M. vitrata* and thrips

in the present study, as well as ICIPE 68 that were ineffective against both fruit fly species. This demonstrates great dynamism in host specificity among fungus-insect interactions that may vary between fungal species and/or isolates, and insect species and/or populations. There is therefore the need to identify potent isolates against *A. craccivora*.

Nevertheless, the present study showed that Nimbecidine® was more effective against aphids compared to *M. vitrata* and thrips. Biopesticide products from the neem tree affect insect biological activities including feeding and oviposition, and also have an impact on regulation of insect growth, moulting and pupation (Murugan and Vanithakumari, 2009) and one or a combination of these actions may have resulted to its high efficacy on *A. craccivora*. Several other studies have demonstrated the efficacy of neem products against different aphid species including the cowpea aphid, *A. crassivora* (Dimetry and El-Hawary, 1995; Baidoo and Agbonu, 2012). Other aphid species susceptible to neem include the soybean aphid, *Aphis glycines* Matsumura (Kraiss and Cullen, 2008), and the cotton aphid, *Aphis gossypii* Glover (Bayhan *et al.*, 2006; Yi *et al.*, 2012; Pinto *et al.*, 2013), among others.

The reason for the poor efficacy of Nimbecidine against *M. vitrata* is not known. However, studies from elsewhere have reported variations in efficacy of neem products against this pest. For example in Taiwan, Biofree–I® did not cause the mortality of the pest, while a different formulation in Thailand (Thai neem 111), was effective with a median lethal concentration of about 2300 ppm against *M. vitrata* (Srinivasan *et al.*, 2012)). Effects of neem may vary among insect species and life stage of the same insect species, a phenomenon more common with antifeedant effects, whereas insect growth disruption and adult sterility are more consistent (Mordue (Luntz) *et al.*, 1998). It is possible that different populations of *M. vitrata* may respond differently to the active ingredient of the biopesticide influencing these processes and behaviours.

In economic terms, the present study revealed that Karate® offers better economic returns on investment followed by Campaign® while Nimbecidine® offers the least returns. Higher returns associated with chemical pesticides have been reported elsewhere (Karungi *et al.*, 2000; Nabirye *et al.*, 2003; Manisegaran *et al.*, 2011; Narasimhamurthy and Keval, 2013), although increased frequency of pesticide application resulted in decreased marginal returns (Karungi *et al.*, 2000; Nabirye *et al.*, 2003). The marginal returns, however only consider direct, short term benefits and costs associated with the treatments, yet some treatments could have direct and indirect, medium and long term costs and benefits. For example chemical pesticides have short, medium and long term negative impact on human health, domestic animals, aquatic life, non-target beneficial organisms including pollinators and natural enemies of insect pests, and the environment at large (Pimentel *et al.*, 1992; Ton, 2000; Chopra *et al.*, 2005; Murugan and Vanithakumari, 2009).

6.6 Conclusion

The present study demonstrates that Karate® is a highly effective pesticide for protecting the cowpea crop against pest damage translating into increased yield. The commercial mycopesticide, Campaign® is effective against *M. vitrata* and thrips, but ineffective against aphid, whereas Nimbecidine® is more effective against aphid and fairly effective against *M. vitrata* and thrips. The observed pesticide efficacies translate into economic gains with Karate® yielding the highest return on investment followed by the mycopesticide, Campaign® while Nimbecidine® offers the lowest returns. Based on results of this study, Campaign® which ranked second after Karate® in terms of pest damage suppression, yield increment and economic returns, is hereby recommended for consideration as a key component of IPM package for cowpea. Future research should explore possibilities of enhancing the efficacy of Campaign® against aphids, and also establish its efficacy against other important pests of cowpea. Additionally, the feasibility

of combining the Campaign® and Nimbecidine® for possible improved efficacy against key insect pests of cowpea could also be explored.

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Discussion

Microbial control, including the use of EPF, has been proposed as an environmentally acceptable alternative to synthetic insecticides in the management of arthropod pests (Lacey and Siegel, 2000; Wraight *et al.*, 2007; Zimmermann, 2007; Shahid *et al.*, 2012; Shapiro-Ilan *et al.*, 2012; Vega *et al.*, 2012). Identifying potent isolates of EPF that are efficacious against different populations of *M. vitrata* is a critical step towards a successful IPM programme for this pest. Fungal virulence, speed of kill and, amenability to mass production are some of the important parameters required for selecting candidate isolates for development as biopesticide (Butt and Goettel, 2000; Wraight *et al.*, 2007; Vega *et al.*, 2009, 2012; Shahid *et al.*, 2012; Shapiro-Ilan *et al.*, 2012). Environmental factors such as temperature, solar radiation, relative humidity, rainfall, and abiotic factors, are known to interact with pathogen and insect host factors to influence the efficacy of EPF (Inglis *et al.*, 2001; Cory and Hoover, 2006; Wraight *et al.*, 2007; Cory and Ericsson, 2010; Jaronski, 2010; Vega *et al.*, 2012).

The present study was undertaken with the aim of evaluating the use of entomopathogenic fungi, *M. anisopliae* and B. *bassiana* for management of *M. vitrata* on cowpea. The study specifically sought to identify and select candidate isolates of EPF that are highly pathogenic to *M. vitrata.* The study also examined the effect of temperature on germination, radial growth and pathogenicity of selected EPF to *M. vitrata* besides assessment of the role of host plant species on the pathogenicity of key isolates of EPF to *M. vitrata.* Performance of the selected EPF in the management of *M. vitrata* on cowpea in the field wss also evaluated.

Results from this study show that all the isolates screened, including 14 of *M. anisopliae* and six of *B. bassiana*, were pathogenic to *M. vitrata*. *Metarhizium anisopliae* isolates were generally more virulent than *B. bassiana* isolates, with isolates ICIPE 18 and ICIPE 69 outperforming the rest. Further screening tests revealed that aqueous and oil formulations of these two isolates were virulent against the egg and larval stages of *M. vitrara*. The adult moths were highly susceptible to dry conidia of the two isolates delivered by direct contamination, incurring 100% mortality while horizontal transmission from males to females only caused sub-lethal effects of reduced fecundity and longevity.

Differences in virulence between fungal species and isolates of the same fungal species are always a common phenomenon in almost all pathogenicity screening studies involving different developmental stages of insect species. Some examples include different isolates of *M. anisopliae* and *B. bassiana* against larvae of the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) (Gindin *et al.*, 2006), the sweet potato weevil, *Cylas puncticollis* Boheman (Coleoptera: Curculionidae) (Ondiaka *et al.*, 2008), and adult fruit fly species, *Ceratitis capitata* (Weidemann), *C. rosa* var. *fasciventris* Karsch and *C. cosyra* (Walker) (Diptera: Tephritidae) (Dimbi *et al.*, 2003). A pathogen that infects several host species in an ecosystem has a better chance of contacting susceptible hosts, thus increasing pathogen population density and disease prevalence (Fuxa, 1987; Shapiro-Ilan *et al.*, 2012). Coincidentally, the two isolates, ICIPE 18 and ICIPE 69, identified in this study as being highly pathogenic to *M. vitrata* have also been reported to cause high mortality in other tropical insects (Ekesi *et al.* 1998; Ekesi *et al.* 2002; Dimbi *et al.* 2003; Migiro *et al.* 2010; Ngumbi *et al.* 2011; Niassy *et al.* 2012a). This suggests that although widely distributed fungi could be relatively host-specific as pathotypes, some isolates can be effective against a broad range of insects. It is important that the safety of such fungal isolates with relatively broader spectrum of activity is investigated as part of the process to develop them into biocontrol agents.

The two isolates were tested for mass production potential in four liquid media. Among the four media, the Jenkins-Prior and APU1 media were superior, and a significantly higher yield of inoculums and biomass was observed for isolate ICIPE 69 compared with ICIPE 18. The obtained level of propagule production falls within the range reported by Kleespies and Zimmermann (1992) and Fargues *et al.* (2002). Some other studies however, have reported higher yield of propagules and biomass, which they attributed to higher quantity of sugar in the media (Vidal *et al.*, 1998). Thus isolate ICIPE 69 is considered to have superior mass production characteristics over isolate ICIPE 18 while Jenkins-Prior and APU1 media are considered ideal for mass production of this isolate.

Temperature is considered as one of the most important factors that influence crucial events such as spore germination, host penetration and growth in the host, that determine the efficacy of EPF (Fargues *et al.*, 1997; Ouedraogo *et al.*, 1997; Li and Feng, 2009; Jaronski, 2010). To further compare the best two isolate for suitability as candidate biopesticides, the effect of temperature on germination, growth and virulence of these isolates against *M. vitrata* was studied. Both fungal isolates germinated at the temperatures of 20 – 35^{o}C while slow growth was recorded at 15^{o}C. Fungal growth occurred at all temperatures, although extreme temperatures (15 and 35^{o}C) resulted in the lowest growth rates. These results are comparable with those reported for these isolates in thermotolerance studies involving different insect species by Ekesi *et al.* (1999) and Dimbi *et al.* (2004). For virulence, optimum temperatures were 30^{o}C for isolate ICIPE 18 and 25^{o}C for ICIPE 69. Mortality due to these isolates generally increased with increasing temperature up to the respective optima of 30^{o}C and 25^{o}C for isolate ICIPE 18 and ICIPE 69, respectively. The thermotolerance of EPF is influenced by the geoclimatic origin of the fungal isolates, although cases of weak or no correlations between the geographical origin and thermal characteristics have also been reported (Fargues *et al.*, 1997). The two fungal isolates tested in this study originated from the Democratic Republic of Congo, which falls within the tropics. Host infection by fungal pathogens is positively correlated

with temperature, but the optimum temperature for fungal development and that of disease development are not necessarily the same (Fargues *et al.*, 1992; Inglis *et al.*, 2001; Vega *et al.*, 2012).

Host plants are considered as one of the key environmental factors that influence the susceptibility of insect hosts to entomopathogens. Plant effects in insect-pathogen relationships can be direct or indirect or both (Butt and Goettel, 2000; Cory and Hoover, 2006; Wraight *et al.*, 2007; Cory and Ericsson, 2010; Vega *et al.*, 2012). The present study assessed host plant effects on virulence, colony forming units (CFU), germination, and persistence. *Maruca vitrata* larval mortality varied between the fungal isolates, and across the three host plants (*V. unguiculata*, *C. cajan* and *P. vulgaris*), with isolate ICIPE 18 being more virulent on cowpea and ICIPE 69 on *P. vulgaris*. *Cajanus cajan* induced 3 – 4 times fewer CFU for isolate ICIPE 69, compared to *P. vulgaris* and cowpea. The fewer CFU of isolate ICIPE 69 exposed to *C. cajan* flowers corresponded with low virulence of the same fungal isolate on the same host post plant, suggesting the presence of antifungal substances in *C. cajan*. Results from persistence bioassay revealed that the treatment factors namely host plant, fungal isolate, and time, singly or through their interaction, affected the persistence of *M. anisopliae*. More spores were recovered from *C. cajan* compared to *P. vulgaris* and cowpea immediately after spraying, but the number reduced significantly to about less than 10% of the originally recorded number of CFU for all the two isolates across the three host plants. The decline rate was considered to be higher in *C. cajan* since this plant had a significantly higher number of CFU than the other two plants at day zero. These differences are attributed to morphological and/or biochemical differences between plants (Wagner, 1991; Dahlin *et al.*, 1992; Oghiakhe *et al.*, 1992; Marley and Hillocks, 2002; Nix *et al.*, 2015).

Indeed, variability in type and density of trichomes has been reported in varieties of *P. vulgaris* (Dahlin *et al.*, 1992; Park *et al.*, 1994), *C. cajan*, and wild *Cajanus* spp. (Romeis

et al., 1999; Sunitha *et al.*, 2008), and *V. unguiculata* (Jackai and Oghiakhe, 1989; Oghiakhe *et al.*, 1992). Additionally, some types of trichomes (i.e. glandular secreting trichomes) are reported to exude phytochemicals such as terpenes, phenolics, alkaloids or other substances, which complement the plant's chemical defense against pests and pathogens (Wagner, 1991; Wagner *et al.*, 2004). Moreover, previous studies have demonstrated presence of antifungal compounds in *C. cajan*, such as isoflavonoid phytoalexins – hydroxygenistein, genistein, cajanin and cajanol (Marley and Hillocks, 1993, 2002). These could explain the differences between *C. cajan* and other host plants (*P. vulgaris* and *V. unguiculata*) in this study. Additional studies are needed on the nature of these compounds and further validate their role in the development and implementation of biopesticide-based management options for *M. vitrata*.

Field evaluation was undertaken to ascertain the effectiveness of the candidate biopesticides in the laboratory bioassays. Considering the fact that isolate ICIPE 69 was relatively superior to ICIPE 18, the commercial formulation of the former (Campaign®) was adopted for field experimentation, and compared with the commercial biorational Nimbecidine® and the chemical pesticide Karate® as a positive check. Results from the two cropping seasons showed that the biopesticide, botanical and chemical pesticide significantly reduced damage caused by the different insect pest species and the effects were noticeable immediately after treatment application. Karate® was effective against all the three pests, as Campaign® was effective against *M. vitrata* and thrips, while Nimbecidine® was more effective against aphids but less effective against *M. vitrata* and thrips compared Campaign®. The untreated control plots recorded the highest damage levels for the all the three insect species throughout the two cropping seasons. Grain yields as well as marginal returns also followed the pattern observed in pest damage. Thus, Karate® recorded the highest yield while Campaign®, ranked second in yield. On the other hand, Nimbecidine®**,** ranked third, but overall yield from this treatment was still higher than the untreated control. *Maruca vitrata* larvae and the legume flower thrips have cryptic

behavior and spend most of their time inside the terminal leaf buds, and bracts/stipules, flower buds and the flowers (Jackai and Daoust, 1986). Uptake of spores from the plant surface and the higher relative humidity prevailing inside this microhabitat facilitates germination of, and infection due to, the fungal conidia on the insect's cuticle and probably contributed to the higher impact of the biopesticide on these insects. The ineffectiveness of the mycopesticide against aphid is generally unknown but could be attributed to the fact that most EPF are known to be host specific, and as such their isolates vary in their pathogenicity to different insects (Sandhu *et al.*, 2012; Vega *et al.*, 2012; Tiago *et al.*, 2014). Thus, fungus-insect interactions may vary between fungal species and/or isolates, and insect species and/or populations.

7.2 Conclusions

i) Different developmental stages of *M. vitrata* i.e. egg, first, second and fourth instar larvae, and the adult stage are susceptible to EPF. Formulating *M. anisopliae* isolates ICIPE 18 and ICIPE 69 in oil enhances efficacy, especially against the egg stage of *M. vitrata*.

ii) Adult male and female *M. vitrata* are highly susceptible to dry conidia of isolates ICIPE 18 and ICIPE 69 through direct inoculation. On the other hand, horizontal transmission from infected male moths to uninfected females results in sublethal effects of reduced fecundity and longevity.

iii) Isolate ICIPE 69 produces higher concentrations of propagules and biomass in two liquid media, Jenkins-Prior and APU1 than isolate ICIPE 18. Therefore isolate ICIPE 69 holds better mass production potential than ICIPE 18 using the two media.

iv) Both isolates ICIPE 18 and ICIPE 69 are virulent against *M. vitrata* over a wide range of temperatures, suggesting that they can be applied in habitats with such prevailing temperature. The optimal temperatures for growth for isolate ICIPE 18

and ICIPE 69 are 33ºC and 30ºC respectively, and correspond with optimal temperatures for development of *M. vitrata* (Adati *et al.*, 2004). Therefore, both the pest and the fungi can coexist within the same habitat thus achieving optimum pest control.

v) Exposing conidia of isolate ICIPE 69 to flowers of *C. cajan* negatively impacted on the number of CFU and virulence against *M .vitrata*, suggesting the presence of antifungal factors in this plant. Aqueous formulations of isolate ICIPE 18 and ICIPE 69 hardly persist beyond 3 days on treated plants.

vi) Karate® is a highly effective pesticide for protecting cowpea against *M. vitrata*, thrips and aphids, and its application increases yield. Campaign® is also effective against *M. vitrata* and thrips, but ineffective against aphids. An IPM package that includes Karate® and Campaign® may offer acceptable and sustainable economic gains to farmers.

7.3 Recommendations

i) *M. anisopliae* isolates ICIPE 18 and ICIPE 69 are recommended for management of *M. vitrata*; and oil formulation would be ideal for targeting egg and larval stages, and a dry conidial formulation is most suitable for adult control.

ii) Additional studies should be undertaken to develop an autodissemination device for the dry conidia targeting the adult moths.

iii) Two liquid media, Jenkins-Prior and APU1, which are based on cheap locally available raw materials, should facilitate production of a low-cost formulation of *M. anisopliae* isolate ICIPE 69 suitable for used by smallholder cowpea producers. Further studies should be undertaken to explore suitable media for the production of isolate ICIPE 18.

iv) There is need to undertake studies to elucidate factors responsible for fungistatic properties of *C. cajan.* In the process developing microbial pesticides, it is highly recommended to test the compatibility of the candidate fungal isolate and the target host plant(s) to be able to make informed decision on host plants for which the final products should be targeted.

v) Based on the laboratory and field experiments reported here, the commercial product of *M. anisopliae* isolate ICIPE 69 (Campaign®) is recommended as an effective biopesticide for the management of *M. vitrata* and thrips, and should be utilized within the context of IPM on cowpea.

vi) Since Nimbecidine® was most suitable for the management of aphids, this botanical and Campaign® should be appropriately timed in such a manner that interventions targeted at aphids is done with the botanical and management of *M. vitrata* and thrips is carried with the biopesticide to negate the continuous use of Karate for management of these pests in cowpea agroecosystem.

vii) The ability Karate®, Campaign® and Nimbecidine® to manage different categories of cowpea pests should be exploited to develop a comprehensive IPM package that would ensure increased yield and economic returns while minimising negative impact on the environment.

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