FUNGAL ENTOMOPATHOGENS (*METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA*) FOR APHID MANAGEMENT: VARIABILITY IN PATHOGENICITY, SPECIES ATTRIBUTES AND INTERACTIONS WITH OTHER NATURAL ENEMIES

BY

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A THESIS SUBMITTED TO THE SCHOOL OF BIOLOGICAL SCIENCES, UNIVERSITY OF NAIROBI, IN FULFILLMENT FOR REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN ENTOMOLOGY

MAY, 2016

DECLARATION

I, Wakuma Bayissa Hundessa (Registration Number: I80/84682/2012) 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. No part of this thesis may be reproduced without prior permission of the author and/or the University of Nairobi.

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DEDICATION

To my beloved wife Biyeshi Ayansa, our son Kena and Bayissa`s family for their love, support and encouragemet.

ACKNOWLEDGEMENTS

I wish to express my gratefulness to the German Academic Exchange Service (DAAD) the African Regional Postgraduate Programme in Insect Science (ARPPIS) of the International Centre of Insect Physiology and Ecology (*icipe*) for monthly stipend, *icipe* for hosting me and all the support provided to me and, the German Federal Ministry for Economic Cooperation and Development (BMZ) for funding the operational research activities. I am also grateful to the University of Nairobi for providing me the training opportunity and necessary support, and the Jimma University for providing me study leave.

I would like to express my deep gratitude to my *icipe* supervisors, Dr. Sunday Ekesi and Dr. Samira Mohamed for invaluable supervision, support and insightful criticism from the conceptualization to the end of this project. My sincere gratitude also goes to my University supervisors Professor Godwin P. Kaaya and Dr. Maina Wagacha for their invaluable mentorship, support and encouragement throughout the study period. I am grateful to Dr. N.K. Maniania for his constructive criticism and editorial comments through numerous drafts. My sincere gratitude also goes to Mr. Benedict Orindi and Dr. Daisy Salifu for their statistical guidance, Dr. Skilton Robert, Mrs. Lillian Igweta, Lisa Omondi and Mama Margaret Ochanda for their great service. I am also grateful to Dr. Tanga Chrysantus for his invaluable guidance on how to handle the experimental insect and Dr. Fathiya Khamis for her encouragement.

My special thanks also goes to Mr. Pascal Oreng, Gordon Otieno, Sande, Callistus Omondi, Levi Dalton, Peterson Nderitu, Fadhili Emanuel and Jane Wanjiru, Joshua Wambua, Alexander Maina, Daniel Oduor and Joseph Ndeg for all their assistance and Tigist Asefa, Dr. Amanuel Tamiru and Kidist Admasu for their support provided to me during my field experiments at Mbita. I thank all my ARPPIS colleagues, my friends Dr. Benti Deressa, Akalu Dafisa, Fikadu Mitiku, Weyessa Geredew, Chemeda Abdeta and Shitaye Gure for being there whenever I needed them. Finally, I would like to express my gratefulness to my family, parents, brothers and sisters for their support, encouragement and love. Above all my utmost thanks to the Almighty God.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
ARPPIS	African Regional Postgraduate Programme in Insect Science
CABI	Centre for Agricultural Bioscience International
ca	Approximately
CI	Confidence Interval
DAE	Days After Emergence
EPF	Entomopathogenic Fungus
FAO	Food and Agricultural Organization
FL	Fiducial Limit
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Model
ha	Hectare
HCDA	Horticultural Crops Development Authority
icipe	International Centre of Insect Physiology and Ecology
IFAD	International Fund for Agricultural Development
IPM	Integrated Pest Management
m.a.s.l	Meter above sea level
ml^{-1}	Per millilitter
mm	Millimeter
MoA	Ministry of Agriculture
LC_{50}	Lethal Concentration that kills 50% of tested population
LT_{50}	Lethal Time (time taken to kill 50% of tested population)
PDA	Potato Dextrose Agar
rpm	Revolutions Per Minute
SSA	Sub-saharan Africa
spp	Species
TOC	Thomas Odhiambo Campus
UV	Ultra Violet

ABSTRACT

Aphids are regarded as one of the most important pest problems of vegetable crops worldwide. Most vegetable growers in sub-Saharan Africa heavily rely on chemical insecticides for control of aphids. However, the synthetic insecticided have detrimental effects on users, consumers and the environment. Fungal-based biopesticides are being considered as alternatives to synthetic chemical insecticides. This study evaluated the virulence of five isolates of *Metarhizium anisopliae* (Metsch.) Sorok. and three of *Beauveria bassiana* (Bals.) Vuill. against apterous adults of *Brevicoryne brassicae* (L.), *Lipaphis pseudobrassicae* (Davis) and *Aphis gossypii* (Glover). The thermotolerance and conidial yield of the isolates were also evaluated as a prerequisite for strain selection. Three isolates of *M. anisopliae* ICIPE 30, ICIPE 62 and ICIPE 69 outperformed the other fungal isolates, causing mortality of 85-98%, 83-97%, and 73-77%, in *B. brassicae, L. pseudobrassicae* and *A. gossypii*, respectively, 7 d post-inoculation. Isolate ICIPE62 had the shortest LT_{50} values of 2.2, 1.6 and 1.5 d; and the lowest LC_{50} values of 0.3×10^5, 0.1×10^5 and 0.05×10^5 conidia ml^{-1} against *A. gossypii, B. brassicae* and *L. pseudobrassicae*, respectively. Furthermore, ICIPE 62 produced more conidia on the surface of aphid cadavers than ICIPE 30 and ICIPE 69 and showed wider thermotolerance with optimum ranges of 25-30ºC. Compared to other isolates, ICIPE 62 appeared to have a broad temperature range of *in vitro* germination, growth and pathogenic activity against the target pests. Isolate ICIPE 62 was, therefore, chosen for further interaction with other natural enemies of aphids and screenhouse and/or field studies.

Laboratory studies were carried out to investigate interaction between three vegetable-infesting aphids, candidate isolate ICIPE 62 and predatory beetle *Cheilomenes lunata* (Fabricius). At a concentration of 1×10^8 conidial ml^{-1}, the fungus was found to cause mortality of 7.5% to *C. lunata* compared to 2.5% mortality in the control 10 days post-treatment. Female adult *C. lunata* to which fungus-infected aphids were offered as prey did not accept them as food source in non-choice bioassays. However, both starved and non starved *C. lunata* fed on live and dead non-infected aphids. In choice bioassay, a total of 1-3 out of 24 infected non-sporulating aphids (average of 0.1-0.4 aphids per arena) were consumed by 48 h starved *C. lunata* within a period of 60 min, but avoided sporulating cadavers. Foraging adult *C. lunata* enhanced the spread of conidia from infected cadavers to healthy aphids, *A. gossypii* feeding on okra (0.8-15.0% mortality),

B. brassicae (3.3-15.0% mortality) and *L. pseudobrassicae* (0.8-14.2% mortality) on kale plants. Results of this study demonstrate compatibility between *M. anisopliae* isolate ICIPE 62 and *C. lunata,* and could provide a sustainable strategy for effective management of aphids on crucifers and okra cropping systems.

The study further investigated the interaction between isolate ICIPE 62 and two important aphid parasitoids: *Aphidius colemani* and *Diaeretiella rapae* under laboratory conditions to determine if use of the fungus against the aphid hosts had effect on parasitism, emergence, sex ratio, morphomety and longevity of F1 generation of the parasitoids. The results showed normal development of *A. colemani* (upto 54-91% adult emergence) when its host *A. gossypii* was first exposed to the parasitoid and then treated with fungus, and 38-92% when it was treated with fungus and then exposed to the parasitoid after 24, 48 or 72 h. A similar trend was also observed for *D. rapae* (i.e. 53-83% and 43-89% adult emergence) when its host aphids *B. brassicae* was treated with fungus before and after parasitism. Conidial concentration and time of exposure had no significant effect on emergence, developmental time, sex ratio, tibial length, wing length and longevity of F1 generation of *A. colemani* and *D. rapae*. This suggests that both parasitoid species and *M. anisopliae* ICIPE 62 may be used together for the integrated management of aphids as long as fungus applications do not interfere with development of the parasitoids.

The potential of candidate isolate ICIPE 62 was further investigated under screenhouse and field condition. The screenhouse experiment was conducted to determine appropriate conidial formulation as a prerequite for field efficacy test. The results showed that application of conidia of ICIPE 62 formulated in aqueous and oil had negative effect on aphid population growth rate (r_i) on kale and okra compared to the controls in screenhouse experiments. However, conidia formulated in oil was found to be superior over aqueous formulation, causing mortality upto 93.8% and 95% in *A. gossypii* and *B. brassicae,* respectively. Based on these results, oil formulation was chosen and further evaluated under field contions. The efficacy of two conidial concentrations formulated in oil were evaluated under field conditions. Chemical insecticide (Karate®) and water were used as controls. *Metarhizium anisopliae* was

applied at the rate of 1×10^{12} conidia ha^{-1} and 5×10^{12} conidia ha^{-1}, and Karate® was applied at the recommended rate of 17.5 g a.i ha^{-1}.

Weekly applications of the fungus and Karate® were compared for two seasons. In all the trials, aphids density was significantly lower in the fungal and chemical insecticide treated okra and kale plots compared to the control. However, no significant variation was observed in aphids density between Karate® treated and control during the second season. The reduction in the aphids density resulted in yield increament in both crops except that no significant difference was observed for okra during the second cropping season. Moreover, higher proportion of aphids mortality with mycosis was observed in fungal treated plots and few in control plots. These findings have demonstrated the potential of *M. anisopliae* isolate ICIPE 62 in suppression of the three aphid species populations, broad termotolerance ability and compatibility with other natural enemies of aphids. The ICIPE 62 can be used as biopesticide and/or IPM package for the control of *A. gossypii*, *B. brassicae* and *L. pseudobrassicae* in okra and crucifer cropping systems and, reduce reliance on synthetic chemical insecticides and their associated risks.

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Background

Vegetables, in their large variety of types, are grown and consumed widely in sub-Saharan Africa (SSA) and play a pivotal role in the nutrition and improvement of livelihoods of its inhabitants (Nyambo and Verschoor, 2005; Smith and Eyzaguirre, 2007; HCDA, 2009; Shackleton *et al.*, 2009). Of the many vegetables grown, crucifer crops including cabbage (*Brassica oleracea* var. *capitata* L., *B. oleracea* var.*capitata*), kale (*Brassica olerace* L. var. *acephala)* and Swisschard (*Beta vulgaris* var.*cicla*) are among the most important crops grown by over 90% smallholder farmers under a wide range of climatic conditions (Nyambo and Löhr, 2005). The crops are grown for consumption, local and export markets in most of East African countries (Oruku and Ndungu, 2001; Nyambo and Löhr, 2005). Particularly, kale is a subsistence crop produced by smallholder farmers as a main component of diet in Kenya, Ethiopia, Zimbabwe and Mozambique (Löhr and Kfir, 2004).

A number of other traditional vegetables including okra (*Abelmoschus* spp) are also grown in the region with a considerable area under cultivation (Smith and Eyzaguirre, 2007). For example, Nigeria is the second largest okra producer in the world accounting for 23.41% of total production (FAOSTAT, 2012). It is mainly produced for tender green pods consumed fresh or dried. The fresh pod contains viscous fiber which is an important dietary component to reduce cholesterol (Kendall and Jenkins, 2004). In addition to the nutritional values, the mucilage in okra has industrial applications as an adhesive and also medicinal application such as plasma replacement (Benchasri, 2012).

The fresh produce of okra is also exported to European market from various African countries. For example, in 2003 to 2005, the Ugandan volume of export produce to United Kingdom was dominated by okra (Legge *et al.*, 2006). In 2005, okra export accounted for 37% of all Asian vegetables and 4.1% of total vegetable export in Kenya (MoA, 2006). Generally, vegetables have potential to ensure food security in the region and can also feed a steadily growing world population.

Despite the enormous contribution of vegetables, there is little improvement to enhance productivity both in quantity and quality. This is mainly attributed to the numerous constraints that seriously affect production and profitability. Aphids (Hemiptera, Aphididae) are important pests of vegetables worldwide (Remaudie`re and Autrique, 1985; van Emeden and Harringiton, 2007; James *et al.*, 2010; Sæthre *et al.*, 2011). These pests are either monophagous or oligophagous (Pettersson *et al.*, 2007), while others are polyphagous (Blackman and Eastop, 1984). The cabbage aphid, *Brevicoryne brassicae* (L.), mustard/turnip aphid, *Lipaphis pseudobrassicae* (Davis) and green peach aphid, *Myzus persicae* (Sulzer) are the most destructive pests of Crucifer (Oduor *et al.*, 1996; Seif, 2002). In tropical Africa, the melon/cotton aphid, *Aphis gossypii* Glover is also reported from various countries; as one of the most serious insect pests on a broad array of vegetables including okra (Varela and Seif, 2004; Nderitu *et al.*, 2008; Saethre *et al.,* 2011; Abang *et al.*, 2014). Infestations due to aphids on these crops can significantly hamper productivity and quality of the plants through direct feeding, contamination with their sugary secretions, and transmission of viruses (Varela and Seif, 2004; Grzywacz *et al.*, 2010).

1.2. Problem statement and justification of the study

Aphids are destructive insect pests of crucifers and okra crops with a cosmopolitan distribution.Crop infestation by aphids significantly reduce the quality and quantity of produce through direct feeding, contamination of the leaves, heads and fruits with exuviae, sugary secretions, and also transmission of viral diseases (Varela and Seif, 2004; Grzywacz *et al.,* 2010). Additionally, the sticky honeydew excreted upon aphid feeding on plant sap, facilitates sooty mold development on fruits and leaves which deteriorates the quality of produce, which can be rejected in domestic and export markets.

Due to their pest status and negative impact on their host crops, aphids attract substantial chemical pesticide use. There is widespread usage of pesticides by nearly 90% of producers in almost all countries where crucifer and okra crops are grown (Obopile *et al.,* 2008; Macharia *et al.*, 2009; Abang *et al.*, 2014). This is further compounded by the high calendar based application frequency of 3-5 days (Williamson *et al.*, 2008). Moreover, there is limited knowledge among vegetable growers especially in SSA on pesticide handling and use, and the level of toxic residues that can negatively affect human and

livestock health and also reduce lucrative export opportunities (Asfaw *et al*., 2009; Macharia *et al*., 2009; Narrod *et al*., 2009). On the other hand, excessive use of chemical insecticides wipe out potential natural enemies, encourage resistant development in target pests, and increase crop production costs (Aktar *et al.*, 2009; Amoabeng *et al.*, 2013). Strict regulation ofpesticide residues management on fresh produce imposed by the importing countries is also becoming a challenge to compliance with the standard (Legge *et al*., 2006; Mausch *et al*., 2006; Narrod *et al*., 2009).

In general, the excessive usage of insecticide and associated risks have raised food safety and sustainability concerns (Dinham, 2003; Asfaw *et al*., 2009; Karungi *et al*., 2011). Therefore, the present pest control strategies should aim at reducing the use of chemical pesticides. This can only be achieved by exploring acceptable alternative pest control options that can reduce yield loss, environmental pollution, risk on consumers and nontarget organisms, and meet quality standards of local and global market.

Based on present knowled, production and use of entomopathogenic fungi (EPF) as mycoinscticides are safe from both an environmental and mammalian toxicity viewpoint (Goettel *et al*., 2001; Roberts and St. Lager, 2004; Zimmermann, 2007). Moreover, temperature required for growth of most *Metarhizium* spp is below mammalian internal body temperature. However, EPF play significant role in the regulation of various insect pest populations. Thus, EPF are being used as alternative control agents against many insect pests. For example, in Europe and North America, several commercial products based on *Metarhizium anisopliae* (Metsch.) Sorok., *Beauveria bassiana* (Bals.) Vuill. and *Isaria* spp. have been registered for aphid control. They include BotaniGard® and Naturalis-L® (*B. bassiana*-based products), Met52® (*M. brunneum*-based product), Preferal® (*Isaria javanica* (Friedrichs and Bally) Samson and Hywel-Jones-based product) and Vertalec® (*Lecanicillium longisporum* R. Zare and W. Gams-based product) (Kabaluk *et al.*, 2010; Raversberg, 2011; Jandricic *et al.*, 2014). Similar products are not available on the African continent.

The effectiveness of EPF is influenced by a range of factors including pathogen type and host insect as well as environmental factors such as temperature (Benz 1987; Hall and Papierok 1982; Ekesi *et al*., 1999; Inglis et al. 2001; Dimbi *et al*., 2004; Wraight *et al.*, 2007; Vega *et al.*, 2012). Temperature is considered as the most important abiotic

factor as it affects the pathogen (conidial germination, growth), insect development and virulence against the insects (Benz 1987; Fargues and Bon 2004; Maniania and Fargues 1992). Thus, the effect of these factors must be addressed for fungal-based mycoinsecticide product development.

Aphids are also attacked by various arthropod natural enemies including predators such as coccinellids and parasitoids which are widely recognized in the suppression of aphid populations (Obrycki and Kring, 1998; Schellhorn and Andow 2005; Van Emden and Harrington, 2007; Evans, 2009; Giorgi *et al*., 2009). In Kenya, parasitoids (*D. rapae* and *A. colemani*) have also been observed attacking *B. brassicae* and *A. gossypii* in kale and okra cropping system, respectively (S. Ekesi, unpublished).

The availability of natural enemies in the agroecosystem and EPF as biopesticide are, therefore, becoming attractive for incorporation into IPM. However, considerably less information is available on the interaction between natural enemies including coccinellids and EPF (Roy and Cottrell, 2008). It is, therefore, important to address the profound absence of knowledge on their interactive roles. The present study was, therefore, conceptualized to identify virulent EPF against the three aphid species, determine its interaction with environmental factors, such as temperature and better understanding of its interaction with other natural enemies such as predators and parasitoids. Screenhouse and field efficacy evaluation of the candidate biopesticide would also be necessary to validate laboratory results as part of IPM package for the aphid pests on crucifers and okra. The findings of this study should contribute to improved sustainable food security and environmental health through the development of IPM for horticultural crops.

1.3. Research questions

This research was intended to address the following key questions:

1. Are entomopathogenic fungi pathogenic to *Brevicoryne brassicae, Lipaphis pseudobrassicae* and *Aphis gossypii,* and what levels of variability exist in their pathogenicity?
2. Do abiotic factors, such as temperature, influence the pathogenicity of candidate isolates?

3. Does intraguild predation exist between coccinellid and pathogen-infected aphid species; and interactions between entomopathogens, predators and the aphid species influence pathogen dynamics and aphid suppression on caged host plants?
4. Does interaction exist between aphid parasitoids and pathogen-infested aphid species and an aphid parasitoids discriminate between pathogen infected and non-infected aphids?
5. What is the performance of candidate isolates in suppression of *Brevicoryne brassicae* and *Lipaphis pseudobrassicae* on kale and *Aphis gossypii* on okra under screenhouse/field condition?

1.4. Objectives of the study

1.4.1. General objective

The aim of this study was to explore the use of entomopathogenic fungi as a component of aphid management and their intraguild interaction with other aphid natural enemies (predators and parasitoids).

1.4.2. Specific objectives

1. Identify and select candidate isolates of entomopathogenic fungus that show high pathogenicity to *Brevicoryne brassicae, Lipaphis pseudobrassicae* and *Aphis gossypii*.
2. Establish the role of temperature on the virulence of candidate fungal isolates.
3. Assess the interaction between selected fungal isolate and coccinellid aphid predators, and establish whether intraguild predation occurs between aphid predators and pathogen infected aphid and its implications on pathogen dynamics.
4. Assess the interaction between candidate fungal isolate and two aphid parasitoids (*Diaeretiella rapae* and *Aphidius colemani*), and establish whether the parasitoids are able to discriminate between pathogen infected and non-infected aphid.
5. Establish the potential of two formulations/concentrations of selected isolate in suppressing target aphid species in screenhouse and/or under open field condition.

CHAPTER TWO: LITERATURE REVIEW

2.1. General morphology and biology of aphids

Aphids belong to the order Hemiptera in the family Aphididae. Genetically identical individuals can exhibit different phenotypes. They are usually small soft bodied with ovoid and plump shape, and body size ranges from 0.5 - 7 mm depending on the species (Miyazaki, 1987). Aphids exhibit polyphenism usually induced by a number of environmental stimuli (Nijhout, 2003; Braendle *et al*., 2006). The presence of natural enemies can also induce aphids to produce the winged phenotype to escape the enemy (Weisser, 1999; Kunert *et al.,* 2008)

Aphids follow various patterns of complex life cycles and typically consist of several asexual generations alternating with a single sexual generation (Moran, 1992). The life cycle patterns include holocyclic or anholocylic on the basis of presence or absence of sexual reproduction and heteroecious or monoecious on the basis of host alteration (Kennedy and Stroyan, 1959). Parthenogenesis, a phenomenan unique to aphids amongst Hemipterans has been instrumental from an evolution point of view (Blackman and Eastop, 2007). The phenomenon involves the alternation of a series of all female parthenogenetic generations with a single sexual generation; in some cases, the sexual generation is totally absent (Moran, 1992). In temperate conditions, parthenogenetic generations occurred in spring and summer followed by a single sexual generation in autumn that produced overwintering eggs. However, most aphid taxa in warmer parts of the world exhibit an obligate asexual (anholocyclic) life cycle in which only parthenogenetic females occur throughout the year (Blackman and Eastop, 2007). The individual parthenogenetic female gives birth to nymphs, which grow fast and produce in 7–10 days, with about 45 generations per year (Ahuja *et al.*, 2010). This cycle comprises of four immature stages (nymphal instars) which resemble the adults but are smaller in size and usually wingless. The variations within and among aphid species and high propagation rate can complicate their control strategies.

2.2. Distribution of aphids

According to Remaurdié and Remaudière (1997), about 5000 aphid species have been described worldwide. However, only about 100 species are considered to be economically important (Blackman and Eastop, 2000). More than 220 aphid species have been identified in sub-Saharan Africa, of which 40% are endemic and about 45% are exotic species which encompass cosmopolitan or sub-cosmopolitan distribution (Autrique and Ntahimpera, 1994). Of all the species, *B. brassicae, L. pseudobrassicae* and *A. gossypii* are among the most destructive insect pests of vegetables in Africa. *Brevicoryne brassicae* and *L. pseudobrassicae* are believed to have originated from Europe and Asia, respectively (Essig, 1948), however, the origin of *A. gossypii* remains unclear (Blackman and Eastop, 2007).

On many cruciferous crops (e.g. cabbage, kale) in Africa, the cabbage aphid *B. brassicae* is restricted to mid- and high-altitude agroecological zones, while the turnip aphid *L. pseudobrassicae* and *A. gossypii* are restricted to mid- and low-land agroecologies. The three aphid species are widely distributed; of which *A. gossypii* is reported from 42 countries in Africa (http://www.cabi.org/isc/datasheet/6204).

2.3. Host range of aphids

Aphids attack a wide range of host plants including vegetable crops. About 10% of aphid pests are considered to be polyphagous (Petherbridge and Mellor 1936; Blackman and Eastop, 1984), and the rest are either monophagous or oligophagous (Pettersson *et al.*, 2007). This implies that some aphid species are specialized in terms of their host preference. Cabbage aphids, *B. brassicae* are exclusively specialist consumers of cruciferous plants in the family Brassicaceae (= Cruciferae), including important crops such as rapeseed, cabbage, kale, broccoli, brussels sprouts, cauliflower, collard, mustard, rape and turnip (van Emden and Harrington, 2007). It also occurs on a wide range of wild and cultivated hosts within the Cruciferae family (van Emden and Harrington, 2007). *Lipaphis pseudobrassicae* is also a consumer of cruciferous and occasionally feeds on other crops such as beans, beet, spinach, pea, celery, onion, cucumber, watercress, zucchini, carrot, kholrabi, lettuce, tomato and potato (Blackman and Eastop, 1984).

The two aphid species (*B. brassicae* and *L. pseudobrassicae*) are generally considered as crucifer-specialist herbivores capable of developing a chemical defense (myrosinase) against host plant biochemical (glucosinolates) defense (Kazana *et al.*, 2007). On the other hand, *A. gossypii* is extremely polyphagous infesting hundreds of plant species including okra (Blackman and Eastop, 2000; Ng and Perry, 2004). For example, in Benin alone, *A. gossypii* was recorded from 17 cultivated vegetable crops belonging to nine plant families such as Amaranthaceae, Apiaceae, Asteraceae, Brassicaceae, Cucurbitaceae, Fabaceae, Lamiaceae, Malvaceae and Solanaceae; weedy plants in the family Apocynaceae, Asclepiadaceae, Asteraceae, Capparaceae, Commelinaceae, Cucurbitaceae, Euphorbiaceae, Nyctaginaceae, Portulacaceae and Poaceae (Saethre *et al.*, 2011). The presence of wide host range may complicate the control of these aphids.

2.4. Damage and economic importance of aphids

Aphids represent one of the world's major insect pests, causing serious economic damage to a diversity of temperate and tropical crops, ranging from grain crops to vegetables and fruit crops (Blackman and Eastop, 2007).

Generally, aphids cause damage to their host plants by sap removal, transmission of plant diseases and contamination through honeydew production (van Emden and Harrington, 2007). Both adults and nymphs feed by piercing plant tissue with their needlelike mouthparts (stylet) and sucking water and nutrients from the phloem system of the plant, causing leaves to curl or twiste inwards with aphids on growing tips and on tender leaves (Miles, 1999; Mossler, 2005). On the other hand, hypersensitive reaction in host plants in response to feeding of aphids may cause necrosis (Miles, 1999).

Feeding damage by aphids can cause considerable yield losses of up to 100% if no control measures are applied in crucifer (Nyambo and Löhr, 2005; Saethre *et al.* 2010; Waiganjo *et al.,* 2011). A study by Sithanantham *et al.*, (1997) also showed that yield losses due to insect pests on okra in Nguruman and Muhaka, Kenya for which *A. gossypii* is regarded as one of the key pests was estimated at 24-40% and 15-24%, respectively.

In addition to feeding damage, aphids cause indirect damage through transmission of important plant diseases. For example, *B. brassicae* is known to transmit 23 plant viruses, of which *Cauliflower mosaic virus* (CaMV) and *Turnip mosaic virus* (TuMV) are known to occur in tropical Africa and can cause substantial reduction in cabbage production (Spence *et al.*, 2007). *Aphis gossypii* too can transmit more than 50 plant viruses causing symptoms that impair vegetable quality and yield (Spence *et al.,* 2007), but the true impact on crop losses have not been quantified.

Aphids also contaminate plants by their honeydew secretion which promotes the growth of sooty moulds (Natwick and Laemmlen, 1993). The presence of sooty mold results in blackening of the leaf that interferes with photosynthetic activity (Elmer and Brawner, 1975; Natwick and Laemmlen, 1993). Moreover, the honeydew secreted by aphids serves as source of food for ants which provide protection for the aphids from potential natural enemies (Gross, 1993; Styrsky and Eubanks, 2007; Nielsen *et al*., 2010).

2.5. Strategies of controlling aphids

2.5.1. Chemical control

A wide range of pests including aphids attack okra and crucifers and require rigorous management. Chemical control is the most widely used by vegetable farmers in most SSA for the control of insect pests including aphids (Ntow *et al.*, 2006; Williamson *et al.*, 2008; de Bon *et al.*, 2014). The routine spraying of these chemical insecticides aimed at reducing crops loss has led to a risk consumers and producers, cause disruption of nontarget species, accumulation of pesticide residues in the environment, and food and feed, and also resistance development in pest species (Dinham, 2003). For example, Koo *et. al.* (2014) reported resistance development in field population of *A. gossypii* to neonicotinoids, pyrethroids, carbamates, niacins and sulfoxamines in Korea. Ahmad *et al.* (2003) also reported high degree of resistance in field population of *A. gossypii* to seven pyrethroid insecticides in Pakistan and Nibouche *et al.* (2002) in Cameroon. Aphids acquire resistance to insecticides through target site insensitivity and through increased production of the affected enzymes (Sun *et al.*, 1987).

Although choice of chemical insecticides can be considered as an option, cost and its availability is of major concern for resource poor farmers in developing countries. For

instance, Brigade® (bifenthrin) is the insecticide of choice in Brassicae integrated pest management (IPM), however the cost limits its usage by resource poor farmers (Grzywacz, 2002).

2.5.2. Host-plant resistance

Although use of resistant cultivars is an alternative to chemical insecticides, there are insufficient cultivars to combat aphid damage in Brassica crops (van Emden and Harrington, 2007; Kennedy, 2008). Previous attempts with conventional breeding techniques have failed to transfer the desired resistance trait in crucifer, but it is thought that genetic engineering mediated transfer of resistance genes can be appropriate (Bhatia *et al.,* 2011). However, such high-tech assisted resistance breeding may not easily benefit farmers in the developing countries. Therefore, this further suggests that use of chemical insecticides still remains the most popular control optionin developing countries. In case of okra, limited effort has been devoted towards the development of aphid resistant varieties. Recently, Abang *et al.* (2014) evaluated 160 varieties of okra from different parts of the world for resistance against *A. gossypii* for use in sub-Saharan Africa. The authors reported that three accessions were found to be resistant to *A. gossypii*. However, these promising varieties are still under development and in limited quantities considering the scale of okra production in the region.

2.5.3. Biological control

The use of biological control is not only efficient in suppressing pest populations, but also considered as a sustainable and eco-friendly pest management strategy that can minimize reliance on synthetic pesticides. It involves use of living organisms (bio-agents) such as predators, parasitoids and pathogens for the management of arthropod pests (DeBach and Schlinger, 1964). Major aphids attacking natural enemies belong to order Dermaptera, Neuroptera, Coleoptera, Hymenoptera and several pathogenic microorganisms (Müller and Godfray, 1999; Völkl *et al.,* 2007). These bio-agents are well known to eliminate or reduce the aphid populations through predation, parasitism and causing diseases (Müller and Godfray, 1999).

2.6. Natural enemies

2.6.1. Predators

Most aphid predators belong to the families Coccinellidae, Syrphidae, Ceccidomyiidae, Chrysopidae, and Anthocoridae. The most common predators of aphids are adult and larval lady beetles, green lacewing larvae, and syrphid fly larvae (Völkl*, et al.,* 2007). Generalist coccinellids have been used successfully for the biological control of several arthropod pests including aphids (Symondson *et al*., 2002; Brown, 2004; Völkl*, et al.,* 2007; Abd-Rabou, 2008). Both larvae and adult stages of Coccinellid feed on the same type of prey species (Völkl, *et al.,* 2007).

Laboratory studies on behavioral aspects of coccinellid showed that predation of pathogen infested aphids or foraging near to pathogen attacked aphids (Roy *et al.,* 2001; Pell and Vandenberg, 2002; Roy *et al,* 2008; Labbé *et al,* 2009; Zaki, 2011) may assist in transmission of entomopathogenic fungi (EPF) as they are coexisting and sharing the same prey. However, their interaction is not well understood because of their diversity. Thus, better understanding of the possible interaction between these agents could provide a new insight for pest management.

2.6.2. Parasitoids

Aphids are attacked by over 600 species of hymenopteran parasitoids (Hymenoptera: Braconidae) (Mackauer and Stary, 1967). Aphid attacking parasitoids belonging to the subfamily Aphidiinae, are solitary endoparasitoids (Stary, 1970) and an adult female usually oviposit a single egg into the bodies of an aphid host. After hatching from the egg, the larvae feed on the tissues within the aphid`s body thereby killing it (Polaszek, 1986). The dead host becomes ‘mummy’ consisting of the hardened exoskeleton of the aphid; the parasitoid larva pupates inside the ‘mummy’ and emerges as an adult (Colfer and Rosenheim, 2001).

Diaeretiella rapae (M’Cintosh) is reported to be the primary and most effective parasitoid of *B. brassicae* (Pike *et al*., 1999) and causes a considerable level of parasitism of up to 75% (CABI, 2007). *Aphis gossypii* is also attacked by several species, most importantly by *Aphidius colemani* Viereck (Takada and Tada 2000; Bolckmans and Tetteroo, 2002; van Lenteren, 2003; Yano, 2006).

The importance of parasitoids in the biological control of aphids has been reported on several occasions; however, the use of broad-spectrum chemical insecticides can cause acute toxicity and interfere with several developmental and survival strategies that hinder their contribution in sustainable crop production (Desneux *et al.,* 2007). Previous studies has been reported the effects of chemichal pesticides on larval development, adult longevity, fecundity, sex ratio, feeding and oviposition behaviors among other factors (Desneux *et al.,* 2007; Garcia, 2011). Such adverse effects can be initiated through direct exposure of the parasitoids to spray droplets, chemical residues on crop foliage or when feeding on chemical contaminated nectar or honeydew (Longley and Stark, 1996). This further suggests the need for developing alternative compatible aphid control options as an IPM package. Biopesticides including mycoinsecticides are among acceptable alternative to synthetic chemical insecticides (Thakore, 2006; Srivastava *et al.*, 2009). However, parasitoids and EPF attacking aphids in the natural environment can influence one another as they compete for the same host resource. Better understanding of their interactionis crucial in designing an effective IPM package for the management of aphids.

2.6.3. Entomopathogens

Bacteria, viruses, fungi, nematodes and protists comprise the major groups of arthropod-pathogens used for biological control. Among these, entomopathogenic fungi (EPF) are one group of microbial pathogens that naturally occur in arthropod pests and induce diseases in the infected host (Wraight and Hajek, 2009). At least 700 fungal species from various taxonomic groups are known to attack and induce epizootics in arthropod pests (Roberts and Humber, 1981; Hajek and St. Leger, 1994; Hajek, 1997; Vu *et al.*, 2007).

The most common insect pathogenic fungi belong to the orders Entomophthorales and Hypocreales. Hypocreales including, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin have received considerable attention in biological control of arthropod pests. Agustino Bassi (1773–1856) was the first to demonstrate that *B. bassiana* was the causal agent of silkworms disease called “white muscardine disease” (Roberts and Hajek, 1992). Later, Elie Metchnikoff (1845–1916) conducted studies on diseases of wheat cockchafers “green muscardine” and identified

M. anisopliae as a causal agent (van Lenteren, 2007; Vega *et al.*, 2009; Roy *et al.,* 2010).

Beauveria is known to have numerous characteristics that have positioned it as one of the best agents that is widely used in research on fungal insect pathology (Steinhaus, 1963) and as biological control agent (Ferron, 1978; Ferron *et al.,* 1991). These characteristics include its cosmopolitan distribution, easy recognition and frequent appearance in nature (Vega and Blackwell, 2005). Some of these characteristics also apply for *Metarhizium*. For example, anamorphic (asexual) lineages of *Metarhizium* are believed to be dispersed from Southeast Asia (Fig. 1) and from this centre most of them have become cosmopolitan (Bidochka and Small, 2005).

Most importantly, both *B. bassiana* and *M. anisopliae* are known to have a wide host range; however, it is recognized that both genera contain a range of individual isolates or pathotypes that can exhibit a restricted host range (Inglis *et al.*, 2001). For biopesticide development and use, strains already existing in the region of use should be prioritized due to the fact that the introduction of exotic strains could be ecologically problematic (Bidochka, 2001). Technical aspects of biopesticide development, such as the selection of strains with rapid kill, mass production potential and formulation type are also important factors. Moreover, long-term stability and consistent efficacy under field conditions (Vega *et al.* 2009), and the role of environmental factors should be considered for fungal-based biopestcide development.

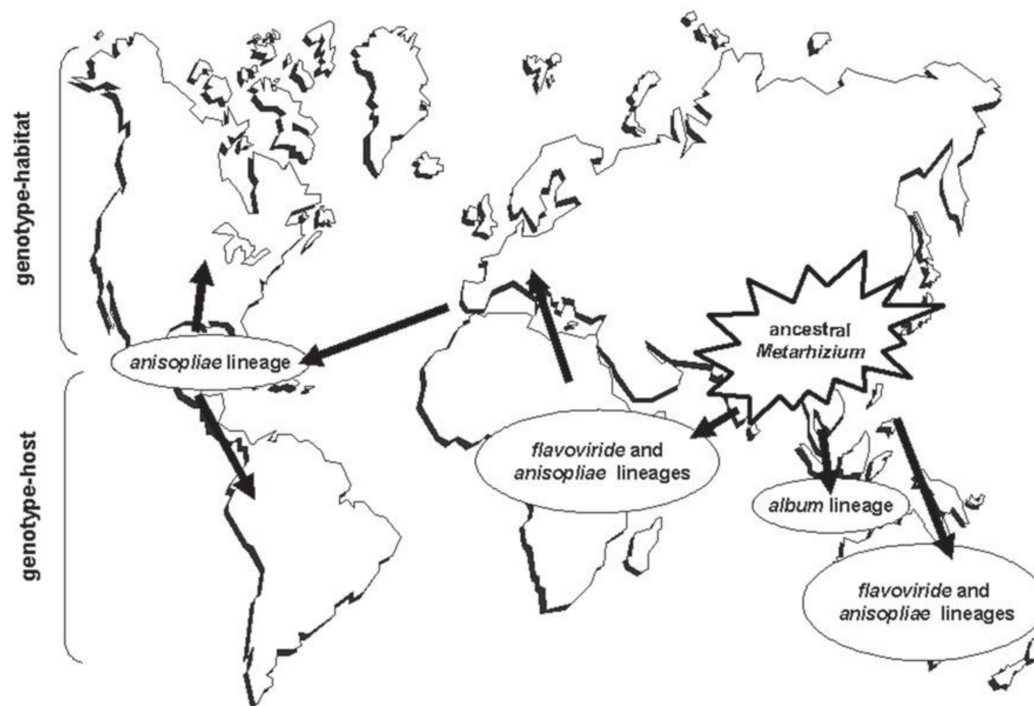

Figure 1. Hypothetical global routes of distribution of *Metarhizium*.
Source: Vega and Blackwell (2005).

2.7. Status of entomopathogenic fungi in biological control

The importance of EPF in the control of pest and vector insects dates as back as early 1773-1856, the period when Agustino Bassi demonstrate that *B. bassiana* was the causal agent of disease in silkworms. Among more than 170 fungal-based products identified, *B. bassiana* and *M. ansopliae* take highest proportions each constituting 34% (Vega *et al.*, 2012). Several workers reported that *M. anisopliae* has been an effective biological control agent against different species of arthropods (Ekesi and Maniania, 1999; Ekesi *et al.,* 2000; Kaaya and Hassan, 2000; Lomer *et al.,* 2001; Maniania *et al.,* 2001; Vega *et al.,* 2009; Bugeme *et al.,* 2014). Moreover, mycopesticide based research in China and Brazil has been dominated by *B. bassiana* and *M. anisopliae* from the historic past to the recent era (Li *et al.*, 2010). According to these authors, *M. anisopliae*-based mycopesticide are applied on about 750000 ha of sugarcane and 250000 ha of grassland per annum to control spittlebugs in Brazil.

Tremendous development has also been made in the development of *Beauveria* and *Metarhizium*-based mycopesticide in Africa (Table 1). For example, *M. anisopliae* var. *acridum* (Green Muscle®) developed by LUBILOSA programme for the control of locust and grasshopper (Douthwaite *et al*., 2001) and registered in several countries including South Africa, Zambia, Namibia, Sudan and Mozambique (Thomas, 2002; Thomas and Read, 2007). In addition to its effectiveness in controlling the pests, this product was also found to have the lowest environmetal risk compared to all other available products. Moreover, several microbial biopesticides have been registered in South Africa (Moore, 2002; Kabaluk *et al*., 2010) and Kenya (Kabaluk *et al*., 2010; Clark *et al.,* 2011) for the management of some important crop pests. Some strains of *M. anisopliae* have also been developed as biopesticides at *icipe* in collaboration with the Real IPM Company (Kenya) Ltd. These commercial products include: *Metarhizium anisopliae* 69 (Campaign®) to control thrips, weevils, whiteflies and mealy bugs; and *Metarhizium* 78 (Achieve®) for the control of spider mites (http://www.*icipe*.org).

Table 1. *Beauveria* and *Metarhizium*-based microbial pesticides in selected countries as of 2009/10

Country	Fungus	Product name	Target pests
Kenya	*Beauveria bassiana* GHA	Botanigard, Bio-power	Aphid, diamond black, moth, sucking insect pests
	Beauveria bassiana	Bb Plus, Bb weevil, Sparticu, Green Muscle	Thrips, weevils, whiteflies
South Africa	*Metarhizium anisopliae subsp. acridum IMI 330189*	-	Locust
	Beauveria bassiana	-	*Monochamus alternatus, Dendrolimus punctatus*
	Metarhizium anisopliae	-	Cockroaches, grasshoppers, locusts
	Beauveria bassiana	Myco-Jaal	Coffee berry borer, diamondback moth, thrips, grasshoppers, whiteflies, aphids, codling moth
United Kingdom	*Beauveria bassiana* ATCC 74040	Naturalis L	Thrips ,whitefly, mites
	Beauveria bassiana GHA	Botanigard	Whiteflies, aphids, thrips
	Beauveria bassiana	Beaugenic, Beaublast	Whiteflies, aphids, sucking pests
	Beauveria bassiana ATCC 74040	Naturalis L	Various insects
United States	*Beauveria bassiana* GHA	Mycotrol ES, Mycotrol O, Botanigard 22WP, BotaniGard ES	Various insects
	Beauveria bassiana HF 23	balEnc	House fly
	Metarhizium anisopliae F 52	Tick-Ex	Ticks and grubs
	Metarhizium anisopliae	BioCane Granules	Grey-backed cane grub (scarabs)
Australia	*Metarhizium anisopliae subsp. Acridum*	Green Guard	Locusts and grasshoppers
	Metarhizium flavoviride	Chafer Guard	Redheaded pasture cockchafer
	Beauveria bassiana HF 23	Balance	House flies
Canada	*Beauveria bassiana* GHA	Botanigard	Aphid, leafhoppers, plant hoppers, mealybug, plant bugs, scarab beetle, thrip, weevils, whitefly
	Metarhizium anisopliae F 52	Met52	Black vine weevil
Brazil	*Beauveria bassiana*	Boveril PL 63	Coleoptera (Curculionidae), Acari (Tetranychidae)
	Metarhizium anisopliae	Biotech, Metarril E9,Metarril 1037	Hemiptera (Cercopidae), Acari (Ixodidae

Source: Kabaluk *et al*.(2010). www.IOBC-Global.org.

2.8. Factors influencing efficacy of entomopathogenic fungi

2.8.1. Host and pathogen factors

Since EPF has been recognized as a causal agent of arthropod diseases, several works investigated the infection process and host defense mechanisms (Boucias, and Pendland, 1991; Hajek and Leger, 1994; Thomas and Read, 2007; Shahid *et al.,* 2012; Ortiz-Urquiza and Keyhani, 2013). The infection process involves the following basic steps (i) attachment of the conidia to the potential host (ii) host recognition and enzyme activation reactions (iii) conidia germination (iv) penetration using penetration structures (germtube and appressorium) (v) multiplication in the haemocoel of the susceptible host (vi) host death and (vii) outgrowing the dead host and production of new infective conidia (Fig. 2).

Infection route of EPF may vary among different groups. For example, Entomophthoralean infect mainly through direct penetration of host cuticle by large sticky conidia, while Hypocrealean (e.g. genera *Beauveria* and *Metarhizium*) infect by small conidia through penetration structures and often mediated by specific proteins (Cole and Hoch,. 1991). On the other hand, some genera (e.g. *Ascosphaera*) infect the host through the gut epithelium after ingestion. Finally, successful infection leads to proliferation of the EPF through hyphal growth and sexual ascospores in hypocreales, protoplasts and asexually through conidia in entomophthoralean (Boomsma *et al.*, 2014) (Fig. 2)

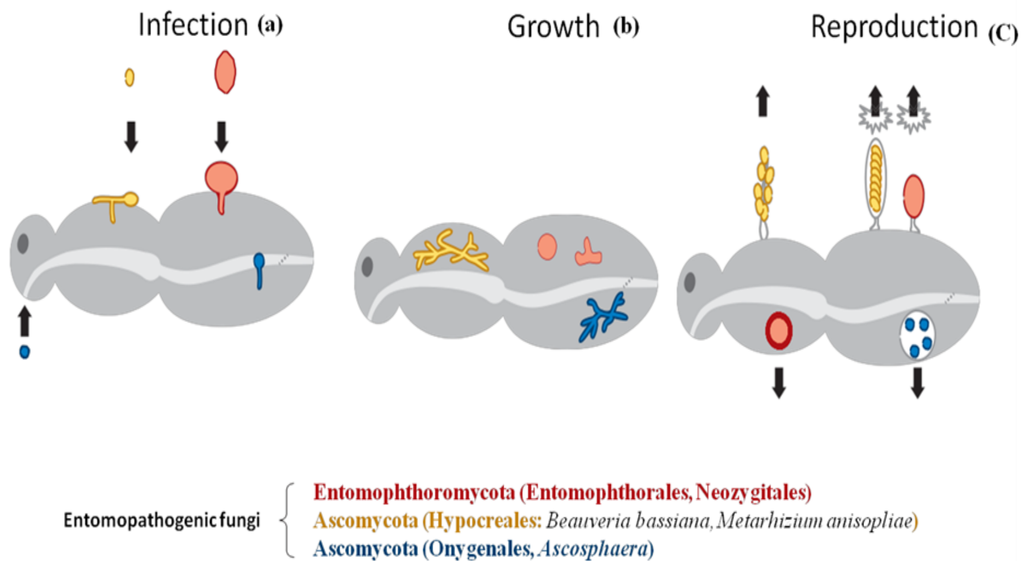

Figure 2. Illustration of infection routes, proliferation and dispersal of entomopathogenic fungi. Source: Boomsma *et al.*(2014).

In fig. 2 above, the downward arrows denotes penetrating the cuticle directly by small conidia of hypocrealean and large stickey conidia of entomophthoralean, and upward arrow from the bottom denotes spore of *Ascosphaera* orally enter (a). Proliferation of hypocreales and onygenales through hyphal growth, and entomophthoralean fungi through protoplasts without cell walls (b). Upward and downward arrows at left denote passive release of hypocreales and downward arrow at right denotes *Ascosphaera* sexual spores that are passively released during the reproduction pahse (c).

In response to attack by natural enemies including EPF, most arthropods employ several defense mechanisms such as structural, behavioral, ecological, physiological and genetic mechanisms. Most importantly, behavioral response induced in response to infection include, induced fever, reduced or increased metabolic activity, reduced response to semiochemicals and changes in reproductive behavior (Roy *et al.,* 2006).

The fungal pathogen must overcome the immune system of the host for nutrient acquisition and further proliferation (Vega *et al.*, 2012). Virulent

pathogens usually produce surface active enzymes (e.g. hydrophobins, Mad adhesions) and secondary metabolites to overcome the host immune response and antimicrobial activities from symbiotic microorganisms (Ortiz-Urquiza and Keyhani, 2013) (Fig. 3). In the last stage of infection, some strains of *Metarhizium* produce toxic substances, such as destruxin in the insect hemocoel (Amiri-Besheli *et al.,* 2000). Such strains usually grow sparsely and kill their host quickly; in contrast, strains that do not produce destruxin grow profusely and take longer time to kill their host (Samuels *et al.,* 1988).

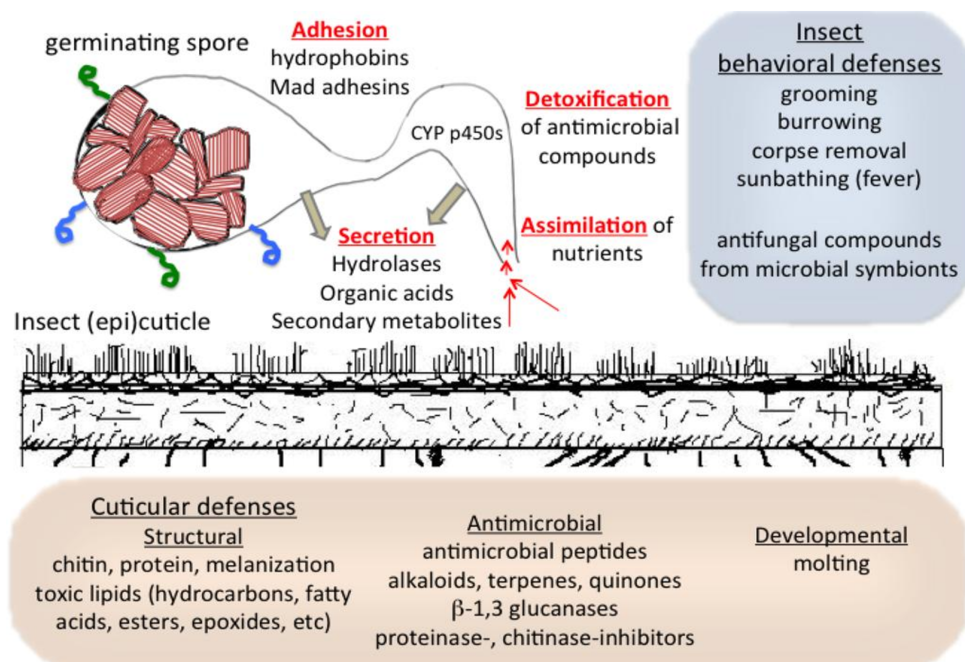

Figure 3. Illustration of host and pathogen defenses. Source: Ortiz-Urquiza and Keyhani (2013).

2.8.2. Environmental factors

Pathogen persistence ability in conditions under which it is to be used is an important attribute for successful biocontrol (Inglis *et al.,* 2001). Several environmental conditions have been shown to have negative effects on their efficacy. Temperature, relative humidity and solar radiation (UV light) are among the most important limiting factors (Inglis *et al.,* 2001; Meikle *et al.,* 2003; Vidal and Fargues, 2007).

2.8.3. Temperature

Temperature is one of the most critical factors influencing EPF efficacy (Benz 1987). It is well documented that temperature influences conidial germination, mycelia growth, sporulation and survival (Inglis *et al.*,1997; Ekesi *et al.,* 1999; Faria and Wraight, 2001; Stacey *et al.,* 2003; Yeo *et al.,* 2003; Dimbi *et al.,* 2004). The optimum temperature required for germination, growth and spore formation for most EPF in the genera of Hyphomycetes is generally between 20 and 30°C (Rangel *et al.*, 2010). The growth of most EPF usually ceases at 37°C (at mammalian body temperature) which is considered an important criteria in mycoinsecticide registration (Inglis *et al.*, 2001). Considerable variability in virulence has also been observed among taxa and within species of EPF in their thermal characteristics. For example, Ekesi *et al.* (1999) and Dimbi *et al.* (2004), noted variability on the virulence level of the same *M. anisopliae* isolate under variable temperatures. However, temperature ranges considered optimum in one region may be lower in other parts of the world (Barriopedro *et al.*, 2011). Thus, there is a need to establish the optimum temperature for successful development and use of the EPF as a pesticide.

2.8.4. Relative humidity

Moisture stress can limit conidia germination and vegetative growth leading to inability to penetrate and recognize susceptible hosts (Hajek and St. Leger, 1994; Ugine *et al.,* 2005).

Although higher relative humidity (RH) is required for conidial germination and the subsequent infection process, some workers reportedthat the infection can occur at as low as 50% RH (Ramoska, 1984; James *et al.* 1998). On the other hand, moisture stress can be mitigated by using an appropriate formulation (Brooks *et al.*, 2004), and also application of irrigation water as it improves the microclimate (Hajek and St. Leger, 1994). For example, successful infection in desert locust was observed at 20-30% RH under field condition using oil-based conidial formulation (Bateman *et al.*, 1993).

2.8.5. Solar radiation

Ultraviolet solar radiations encountered in the field, in particular UV-B spectrum ranges between 285–315 nm, may damage the DNA, RNA, and proteins and others cell constituents (Griffiths *et al.*, 1998; Inglis *et al.*, 2001). The surviving fungal conidia after exposure to UV radiation require long time to recover and restore the germination process (Santos *et al.,* 2011). However, the susceptibility to UV radiation depends on the physiological state of the infective conidia. For example, Braga *et al.* (2001) observed that dormant conidia were generally the most resistant to UV radiation compared to conidia undergoing germination. Fargues *et al.* (1996) also reported variability in sensitivity to UV among taxa, species and strains within species. Incorporating nutrient agar, glycerin and molasses to EPF formulation could also help as protectants (Maniania *et al.* 1993).

2.9. Formulation of entomopathogenic fungi

A number of studies have shown that formulation products acted as a sticker and protectant against various environmental stresses. It has been reported that formulation improves product shelf life, efficacy and other characteristics necessary for application (Wraight *et al.,* 2001). Most often formulation product contains carrier, diluent, binder, dispersant, UV protectants and virulence-enhancing factors. For example, Tinopal has been identified as a UV protectant carrier regardless of concentration level (Reddy *et al.,* 2008). On the other hand, Fantesk^{TM} is proven to improve the shelf life of blastospore under cold storage condition (Jackson *et al.,* 2006). The lipophilic nature of insect cuticle favors adherence of oil-based formulation than droplets of water as the oils spread rapidly and carries conidia to areas on insect body (Wraight, *et al.*, 2001). For example, Kaaya and Hassan (2000) reported that high mortality in *Rhipicephalus appendiculatus* Neumann *and Amblyomma variegatum* Fabriscius followed application of conidia formulated in oil. Brooks *et al.* (2004) also reported improved pathogenicity and thermotolerance of oil-formulated mycoinsecticides than aqueous formulations or dry unformulated conidia. In other studies, the application of oil based formulation of *M. anisopliae* , *B. bassiana* and *V. lecanii*

against sucking pests including aphids showed significant yield increment in okra (Naik and Shekharappa, 2009).

2.10. Intraguild interaction

The word "guild" is defined by Root (1967) as "a group of species that exploit the same class of environmental resources in a similar way." However, Polis and Myers (1989) used the term "guild" in a broader sense as "all taxa in a community that use similar resources (food or space) and thus may compete, regardless of differences in tactics of resource acquisition". Polis and Myers (1989) further elaborates that "interactions between species are usually categorized as competition (- -), predation/parasitism (+ -), mutualism (+ +), commensalism (+0) and amensalism (-0). Intraguild predation is a combination of the first two, that is, the killing and eating of species that use similar, often limiting resources and are thus potential competitors."

The interaction between entomopathogenic microbial organisms and other natural enemies (predators/parasitoids) for prey or other resource acquisition (Fig. 4) is prevalent in nature (Hochberg and Lawton, 1990; Ryalls *et al.*, 2013). Few studies have investigated and quantified the interactions among different types of aphids` natural enemies, and its implication for the control of aphids populations. Accordingly, the possible interactions have been suggested as synergistic or antagonistic, and in some cases not well explained (Roy and Pell, 2000; Mesquita and Lacey, 2001; Pell and Vandenberg, 2002; Pope, *et al.,* 2002; Ekesi *et al.,* 2005; Roy *et al.,* 2006; Baverstock *et al.,* 2009 and Rashki *et al.,* 2009).

Case studies on the interaction between fungal pathogens and coccinellid or aphid parasitoids have revealed the avoidance of fungal infected hosts by ovipositing wasps (Brobyn *et al.*, 1988; Fransen and Van Lenteren, 1994) and some generalist insect predator (Meyling and Pell, 2006). Mesquita and Lacey (2001) also tested the oviposition behavior of *Aphelinus asychis*, development of their progeny and other parameters using *Paecilomyces fumosoroseus* infected host aphids *Diuraphis noxia.* They noted synergistic interaction between *P. fumosoroseus* and *A. asychis,* while an antagonistic relationship was observed between *P. fumosoroseus* and the

host aphid. Baverstock *et al.* (2010) while studying the competition for prey between *C. septempunctata* and *P. neoaphidis* reported that the impact of entomopathogenic fungi was less harmful to predators as compared to parasitoids. Moreover, Roy *et al.* (2001) reported that the aphid predator *Coccinella septempunctata* foraging on plants with aphid cadavers enhanced transmission of fungal pathogen *Erynia neoaphidis* to the healthy aphid populations on the plant. However, there are no similar records for the interaction between EPF (*B. bassiana* and *M. anisopliae*) and *C. lunata,* in addition to the paucity of information for aphid parasitoids (*A. colemani* and *D. rapae*).

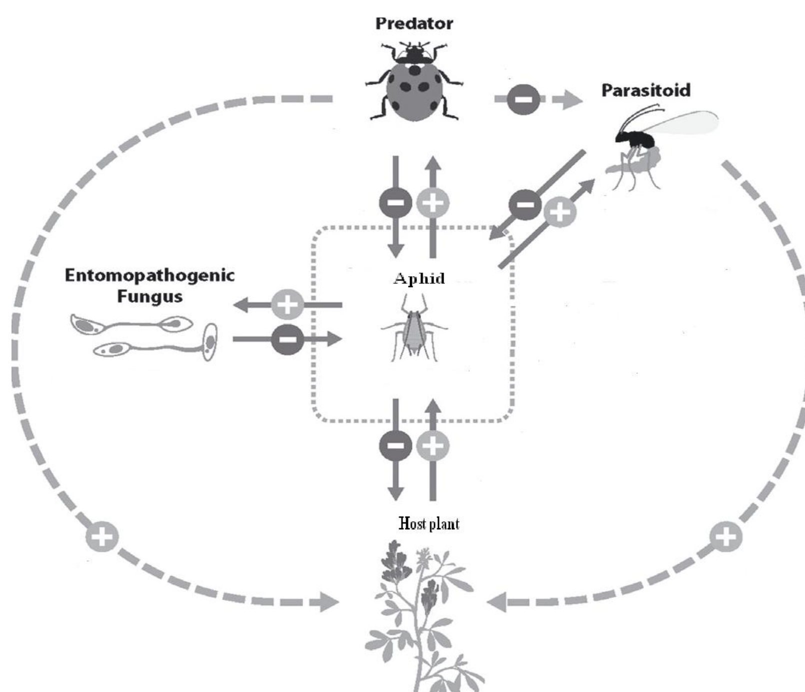

Figure 4. Schematic representaion of interaction among host insects, plants, entomopathogens, predators and parasitoids. Dote arrows denoted indirect interactions whilesolid arrows denote direct interactions. Positive interation are denoted by (+) and negative interactions denoted by (–); modified from Ryalls *et al.* (2013).

CHAPTER THREE: GENERAL MATERIALS AND METHODS

3.1. Experiments under laboratory, screenhouse and field conditions

This study was conducted under laboratory, screenhouse and open field conditions. Each setting is introduced in this chapter and the detailed descriptions are given under the corresponding chapters.

The laboratory bioassays included screening of entomopathogenic fungi (EPF) for their virulence (Chapter 4), thermotolerance study (Chapter 5), interaction between predators and EPF (Chapter 6) and interaction parasitoids and EPF (Chapter 6) were conducted under laboratory condition at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi. Screenhouse experiments (Chapter 8) in *icipe*, Duduville Campus and field experiments (Chapter 9) at the *icipe*, Thomas Odhiambo Campus (TOC), Mbita, at the shores of Lake Victoria, Kenya (00° 25' 949" S, 34° 12' 412" E; 1200 m above sea level). Screenhouse and field study sites were selected on the basis of the availability of research facilities (such as screenhouse and plots).

3.2. Procedure of rearing aphids

Three aphid species namely, *B. brassicae, L. pseudobrassicae* and *A. gossypii* (Fig. 5) were used for laboratory bioassays, screenhouse and field studies. The aphids were obtained from a field population; the two aphid species, *B. brassicae* and *L. pseudobrassicae* from infested cabbage and kale while *A. gossypii* from okra crops and reared in insectary to achieve uniform age groups (Fig.6) for the laboratory bioassays and screenhouse experiments.

Figure 5. Aphis gossypiii infestation on okra (A), Brevicoryne brassicae (B) and Lipaphis pseudobrassicae (C) on kale. Photos: W.H. Bayissa**.**

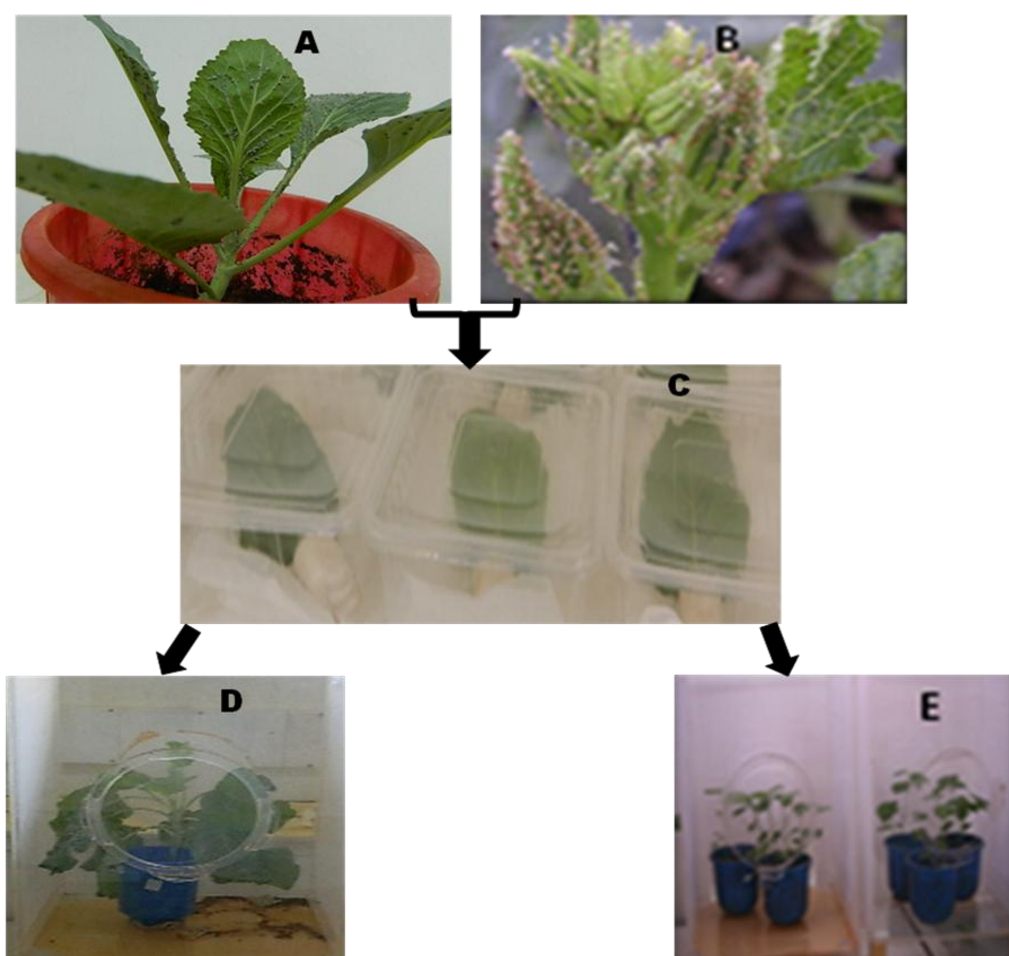

Figure 6. Schematic representation of aphids rearing procedure in insectary for the subsequent laboratory bioassays: mother colony of aphids on kale and okra seedlings (A and B), individual adult aphids removed from mother colony and maintained on detached leave to larviposit (C), first instar of *B. brassicae* or *L. pseudobrassicae* (D) and *A. gossypii* (E) maintained on their respective host until they grow to adult stage (8-10 days) under 12 hour L: D and 26 ± 2°C.

Photos: W.H. Bayissa.

3.3. Fungal cultures and inoculum production

3.3.1. Fungal cultures

Five isolates of *M. anisopliae* (ICIPE 18, 30, 62, 69 and 78) and three *B. bassiana* (ICIPE10, 273 and 279) were obtained from *icipe*'s Microbial Bank and sub-cultured on Sabourad dextrose agar (SDA) for the production of aerial spores for the subsequent laboratory bioassays. Two to three week old cultures were harvested in 10 ml of sterile distilled water containing 0.05% Triton-X100 for use in the laboratory bioassays. In order to maintain the virulence of the fungal pathogens, it was passaged through adult aphids when necessary.

3.2. Production of fungal inoculum

The fungal spores used in screenhouse and field experiments were mass produced on rice grain. First, blastospores were produced in liquid broth (LB) medium containing 15g Yeast Extract, 15g Glucose, 5g Peptone in 500 ml sterile distilled water. The medium was autoclaved for 20 min at 121°C and 15 psi, and then cooled to 28°C before inoculating with fungal cultures. Conidia were harvested from 2-3 weeks old cultures, suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 and vortexed. Spore concentration was adjusted to 1×10^8 conidia ml $^{-1}$ and 5 ml transferred into 50 ml of sterile LB medium in 250 ml flask. The 5 ml spore suspended in 50 ml of sterile liquid medium was incubated in a rotary shaker at 100 oscillations per minute at 25°C for 3-7 days to obtain blastospores . Two kilograms rice substrate was soaked in boiled water in plastic buckets for 10 to 15 min. After draining off the water, the substrate was transferred to sterile polyethylene bags and autoclaved for 60 min at 121°C. The substrate was cooled to 28°C and inoculated with the 3-7 days old culture of blastospores and the bag sealed under aseptic condition. The culture was incubated for about 3 weeks at 20-26°C and 40 - 70% RH. After 3 weeks, the polyethylene bag was removed to allow the culture to dry for 5 days at room temperature (26 ± 2°C) before harvesting conidia (Ekesi *et al.*, 2001). The conidia were harvested and stored at 4°C for further use.

3.4. Selected natural enemies of aphids

Predators: The most prevalent Coccinellid species were obtained from kale and/or okra agroecosystems, identified as *C. lunata*. The adult beetles were maintained on aphid infested kale and/or okra in the insectary (detailed methodology described in Chapter 6).

Parasitoid species: Two aphid parasitoids, *Aphidius colemani* (Viereck) and *Diaeretiella rapae* (McIntosh) (Braconidae: Hymenoptera) were used for this study. Stock cultures of *D. rapae* and *A. colemani* were obtained from the *icipe* insect mass rearing facilities. *Aphidius colemani* was reared on *A. gossypii* and *D. rapae* on *B. brassicae* and *L. pseudobrassicae* or *M. persicae* infesting okra and kale, respectively, in cage (100 x 100cm) for continuous supply throughout the experiment. The adult wasps were fed on honey drops on the roof of the rearing cages (detailed methodology described in Chapter 7).

CHAPTER FOUR: SELECTION OF FUNGAL STRAINS AND EVALUATION OF THE VIRULENT ISOLATE AGAINST APHIDS ON CRUCIFER AND OKRA VEGETABLES

4.1.Introduction

Globally, aphids (Hemiptera: Aphididae) are regarded as one of the most important insect pests of vegetable crops causing damage by direct feeding on plant sap, excretion of honeydew that may favor the growth of molds and transmission of plant viruses (van Emden and Harrington, 2007). In Africa, the cabbage aphid *Brevycorine brassicae* (L.)- restricted to mid- and high-altitude agroecologies, and the turnip aphid, *Lipaphis pseudobrassicae* (Davis), restricted to mid- and low-land agroecologies, cause considerable yield losses of up to 100% on many cruciferous crops (e.g. cabbage, kale) if not controlled (Nyambo and Löhr, 2005; Waiganjo *et al.*, 2011; Sæthre *et al.*, 2011; S. Ekesi and R. Hanna, unpublished).

The cabbage aphid is known to transmit 23 plant viruses, of which *Cauliflower mosaic virus* (CaMV) and *Turnip mosaic virus* (TuMV) occur in tropical Africa and can cause substantial reduction in cabbage production (Spence *et al.*, 2007). In Kenya, survey of farmers perception on insect problems of kale and cabbage revealed that 89-97% of the growers ranked aphids (*B. brassicae* and *L. pseudobrassicae*) as the major insect pest threat on the two crops (Oruku and Ndungu, 2001).

In addition to these two aphid species, the melon/ cotton aphid, *Aphis gossypii* (Glover) is also considered as one of the most serious pest on a broad array of vegetables in tropical Africa. For example in Kenya, yield losses due to *A. gossypii* on okra in Nguruman and Muhaka were estimated at 24-40% and 15-24%, respectively (Sithanantham *et al.*, 1997). *Aphis gossypii* can transmit more than 50 plant viruses causing symptoms that impair vegetable quality and yield.

In many African countries, the management of these aphid species relies heavily on application of synthetic chemical insecticides. Majority of vegetable growers who are smallholders with limited knowledge of pesticide use frequently apply

cocktail of synthetic chemical insecticides (Williamson *et al.*, 2008; Zalucki *et al.*, 2012). This practice substantially elevates production costs, increases health risks to producers and consumers, and most often disrupts the activity of natural enemies that otherwise could contribute to keeping the aphids under control, in addition to the development of resistance to these chemicals (Aktar *et al.*, 2009; Amoabeng *et al.*, 2013). Therefore, exploring non-chemical alternatives for controlling aphids is fundamental to realizing sustainable vegetable production, especially among the smallholder farmers in the region.

Aphids are known to be attacked by a number of natural enemies including EPF which sometimes cause epizootics in their populations (Wraight and Hajek, 2009). Among the EPF, Hypocreales of the anamorphic genera (*Beauveria, Metarhizium, Isaria,* and *Lecanicillium*) are best suited for development as biopesticides compared to Entomophthorales which are highly specialized and difficult to mass produce. They involve parasitic, hemibiotrophic (i.e. phase in insect haemocoel) and saprophytic phases (Wraight and Hajek, 2009; Hesketh *et al.*, 2010) that are amenable to mass production on various organic substrates such as rice, maize and sorghum among others. In Europe and North America, several commercial products based on *M. anisopliae, B. bassiana,* and *Isaria* spp. have been registered for aphid control. They include BotaniGard® and Naturalis-L® (*B. bassiana*-based products), Met52® (*M. anisopliae*-based product), Preferal® (*Isaria javanica* (Friedrichs and Bally) Samson and Hywel-Jones-based product) and Vertalec® (*Lecanicillium longisporum* R. Zare and W. Gams-based product) (Kabaluk *et al.*, 2010; Raversberg, 2011; Jandricic *et al.*, 2014). Similar products are not available on the African continent. There is need, therefore, to identify virulent fungal isolates that could be developed as biological control agents of aphids on vegetable crops in Africa. The objective of the present study was therefore to screen fungal isolates against *B. brassicae, L. pseudobrassicae* and *A. gossypii* for selection of virulent isolates that could be developed further.

4.2. Materials and Methods

4.2.1. Collection and rearing of aphids

The stock cultures of aphids (*B. brassicae, L. pseudobrassicae* and *A. gossypii)* were sampled from cabbage, kale and okra crops in Nyeri, Kenya (0°21'10.69"S 37°5'14.35"E, 1878 m.a.s.l), and Nguruman, Kenya (1°48'22.1"S 36°03'41.2"E, 746 m.a.s.l). The aphids were reared on at least 3 weeks old kale (var. 1000 headed) and okra (var. Pusa sawani) potted plants (4-5 leaves) in the *icipe* insectary at 27-28°C and photoperiod of 12 : 12 L: D. Laboratory-reared colonies were rejuvenated every three months by introducing field populations of the same aphid species to maintain genetic vigor. To obtain insects of the same developmental stage for use in bioassays, apterous adult aphids of each species were removed from laboratory-reared colonies using camel brush and maintained on fresh kale or okra leaves in 90 mm Petri dishes to larviposit for 24 h and adults were removed thereafter. The newly-born nymphs were then transferred to fresh seedlings and maintained until adult stage before they could be used for the bioassay.

4.2.2. Fungal isolates

Fungal isolates used in this study were obtained from the *icipe* Arthropod Germplasm Centre where they were stored at -80°C. The list and origin of the fungal isolates, and the year of their isolation are presented in Table 2. They were cultured on Sabouraud Dextrose Agar (SDA) and maintained at 26 ± 2°C in complete darkness. Virulence of each fungal isolate was maintained by regular passages through the insect host. Conidia were harvested from 2-3 weeks old sporulating cultures (Fig. 7) by scraping using a sterile spatula and suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads (3 mm). The suspension was vortexed for 5 minutes at 700 revolutions per minute (rpm) to break the conidial clumps and ensure a homogeneous suspension. Conidia concentration was quantified using haemocytometer under light microscope. For viability test, concentration of 3×10^6 conidia ml^{-1} was prepared and 0.1 ml of the suspension was spread-plated on SDA using sterile glass rod and three sterile microscope cover slips were

placed randomly on the surface of each inoculated plate. The plates were sealed with parafilm and incubated under complete darkness at 26 ± 2°C. Conidia germination was assessed after 18 h by counting 100 conidia under each coverslip using a light microscope (× 400 magnification).

Table 2. List and origin of fungal isolates used in the study

Fungal species	Fungal Isolate	Origin	Site (Country of origin)	Year of isolation
B. bassiana	ICIPE 10	Soil	Mbita (Kenya)	2002
	ICIPE 273	Soil	Mbita (Kenya)	2006
	ICIPE 279	Coleopteran larvae	Kericho (Kenya)	2005
M. anisopliae	ICIPE 18	Soil	Mbita (Kenya)	1989
	ICIPE 30	*Busseola fusca*	Kendubay (Kenya)	1989
	ICIPE 62	Soil	Matete (D.R. Congo)	1990
	ICIPE 69	Soil	Matete (D.R. Congo)	1990
	ICIPE 78	*Temnoschoita nigroplagiata*	Ungoe (Kenya)	1990

A B

Figure 7. Sporulating cultures of entomopathogenic fungi used in the screening experiment: (A) *Metarhizium anisopliae* (B) *Beauveria bassiana*.
Photos: W.H. Bayissa.

4.2.3. Pathogenicity of fungal isolates against *B. brassicae, L. pseudobrassicae* and *A. gossypii*

Five isolates of *M. anisopliae* and three of *B. bassiana* were screened against the three aphid species. Twenty (20) two-day old apterous adult aphids of each species were transferred on fresh leaf disks (ca. 80 mm diameter) of kale for *B. brassicae* and *L. pseudobrassicae,* and okra for *A. gossypii* in a 90-mm Petri dish. Insects were allowed to settle on the host plants and were then sprayed with 10 ml of aqueous suspension of each fungal isolate at a concentration of 1×10^{8} conidia ml^{-1} using Burgerjon's spray tower (Burgejon, 1956). Air atomizing nozzle with a valve providing a constant airflow under 4 bar pressure fitted to the Burgerjon's spray tower resulting to suspension deposit of approximately 3.9×10^{7} conidia cm^{-2}. Rotating plate fitted underneath of the tower also ensure even distribution of the

suspension on the surface of the insects. The control groups were sprayed with sterile distilled water containing 0.05% Triton X-100. After treatment, embedded leaf discs containing aphids were allowed to dry (for approx. 5 min). The aphids were then transferred to fresh unsprayed surface sterilized leaf disks (ca. 100 mm diameter). Both control and fungus-treated insects were transferred to a humid plastic box (40 × 120 mm) lined with moistened filter paper and incubated at 26 ± 2°C. All the fungal isolates were bioassayed concurrently against the three aphid species and replicated four times over time. Treatments were arranged in a completely randomized block design. Aphid mortality was recorded daily for 7 days post-treatment. The dead insects were surface-sterilized with 70% alcohol and then rinsed thrice in sterile distilled water. They were kept separately in a Petri dish lined with sterile moistened filter paper to initiate mycosis. The later was confirmed using a dissecting microscope (Leica EZ4) at × 20.

4.2.4. Dose-mortality response

Metarhizium anisopliae isolates ICIPE 30, ICIPE 62 and ICIPE 69 outperformed the other fungal isolates in the screening tests and were selected for dose-response mortality assays using the following concentrations: 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 conidia ml^{-1}. Suspensions were prepared as described in section 4.2.2 and different concentrations were obtained through serial dilutions. Groups of each adult apterous aphid species were placed on their respective host plant leaves (kale or okra) as described earlier and were sprayed with 10 ml of each concentration using Burgerjon spray tower (Burgejon 1956). The control groups were sprayed with sterile distilled water containing 0.05% Triton X-100. Twenty apterous adult aphids were used per replicate and per dose for each isolate and aphid species. Test-insects were maintained as described in section 4.2.1 and mortality recorded daily for 7 days. Treatments were randomized and repeated three times. Dead insects were processed following the procedures described in section 4.2.3.

4.2.5. Conidia production on aphid cadaver

Conidial production on the insect cadavers resulting from application of the three virulent isolates of *M. anisopliae* (ICIPE 30, ICIPE 62 and ICIPE 69) was evaluated from the dose of 1×10^8 conidia ml^{-1}. Three mycosed insects were randomly selected from each replicate for each aphid species and used to estimate conidial yield at 3, 6 and 9 d post-treatment. Cadavers were individually placed in a sterile Petri dish and dried at 30 ± 2°C for 30 min in oven, and transferred thereafter into 2.5 ml cryogenic tube containing 1 ml of sterile distilled water containing 0.05% Triton X-100. The tubes were vortexed for 5 minutes to dislodge conidia and conidial concentration from each cadaver was determined using a hemocytometer (Niassy *et al.*, 2012).

4.2.6. Statistical analyses

All data analyses were done using R v2.14.2 statistical software package (R Development Core Team, 2011). Percent aphid mortality were corrected for control mortality using Abbott's formula (Abbott 1925) and then normalized by arcsine square-root transformation (Gomez and Gomez 1984) before analysis of variance (ANOVA). Trial date was included in the model as a factor to control for trial-to-trial differences. Mean comparisons among aphid species, fungal species and/or isolates were made using Tukey HSD test. Data on percent germination of fungal isolates were subjected to GLM using binomial regression analysis. Whenever overdispersion was detected, data were fitted to quasibinomial regression model. Lethal time to 50% mortality (LT_{50}), lethal concentration to 50% mortality (LC_{50}) and slope were estimated by probit analysis. Count data on conidial production was checked for normality and homogeneity of variance using Shapiro-Wilk test before analysis and then were fitted to GLM using negative binomial regression analysis. Negative binomial distribution was preferred for its appropriateness in handling the overdispersed conidial count data.

4.3. Results

4.3.1. Pathogenicity of fungal isolates against aphids species

The spore germination for each isolate was greater than 90% after 18 h incubation at 26 ± 2°C and was considered as acceptable (Table 3). Control mortalities in the initial screening ranged from 15-20% across the three aphid species. Compared to control insects, all fungal treated insects showed external hyphal development and sporulation under moist incubation conditions (Fig. 8 A, B and C).

Table 3. Percentage germination of fungal spores tested against three adult aphid species on SDA plates after 18 h at 26 ± 2°C.

Fungal species	Fungal Isolate	% germination ± SE*
B. bassiana	ICIPE 10	95.5 ±0.3ab
	ICIPE 273	91.4±2.1b
	ICIPE 279	93.1±1.2b
M. anisopliae	ICIPE 18	98.1±0.3a
	ICIPE 30	98.9±0.5a
	ICIPE 62	99.3±0.1a
	ICIPE 69	99.8±0.3a
	ICIPE 78	99.1±0.3a

*Viability of conidia on SDA plates after 18 h at 26 ± 2°C. Means ± SE within a column followed by the same letter are not significantly different by Tukey's HSD multiple range test at 5% level.

At a standard concentration of 1×10^{8} conidia ml^{-1}, there was a significant difference between *B. bassiana* and *M. anisopliae* ($F = 65.2$, $df = 1,69$; $P < 0.0001$) and also among fungal isolates ($F = 14.48$, $df = 7,69$; $P < 0.0001$). There were also significant differences in interactions between fungal species and insect species ($F = 11.29$, $df = 2, 69$; $P < 0.0001$) and between fungal isolates and insect species ($F = 4.69$, $df = 14,69$; $P < 0.0001$) (Table 3). *Metarhizium anisopliae* isolates were more pathogenic to the aphid species than *B. bassiana* isolates, causing mortality ranging from 72.0-98.3%, 74.3-96.8% and 61.9-77.0% in *B. brassicae, L. pseudobrassicae* and *A. gossypii,* respectively, 7 days post-treatment (Table 3).

The lethal time required to achieve 50% mortality (LT_{50}) among aphid species also varied with fungal isolates (Table 4). LT_{50} values varied from 1.6-4.6 days in *B. brassicae*, from 1.5-4.2 days in *L. pseudobrassicae* and from 1.9-4.7 days in *A. gossypii. Metarhizium anisopliae* isolate 62 had the shortest LT_{50} values against the three species of aphids while *B. bassiana* isolate ICIPE 279 had the shortest LT_{50} value against *A. gossypii.* The LT_{50} value for isolate ICIPE 279 was not computed against *L. pseudobrassicae* since mortality was less than 50% after 7 days post-treatment (Table 4).

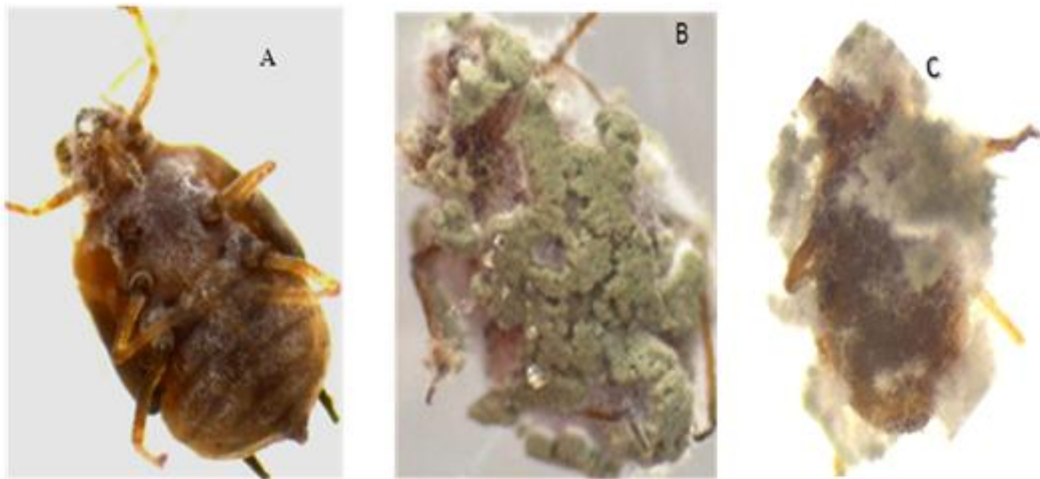

Figure 8. Unmycosed adult of *Brevicoryne brassicae* from control treatment (A) mycosed B. brassicae covered with hyphae and spores from *Metarhizium anisopliae* treatment (B); mycosed B. brassicae covered with hyphae and spores from *Beauveria bassiana* treatment (C) (×20 magnification).

Photos: W.H. Bayissa.

Table 4. Pathogenicity of isolates of *M. anisopliae* and *B. bassiana* to apterous adult *B. brassicae, L. pseudobrassicae* and *A. gossypii* following exposure to 1× 10^8 conidia ml^{-1} after 7 days: percentage mortality and LT_{50} values.

Fungal Species	Isolate	*Brevicoryne brassicae*		*Lipaphis pseudobrassicae*		*Aphis gossypii*	
		% Mortality ± SE	LT_{50} (days) (95% FL)	% Mortality ± SE	LT_{50} (days) (95% FL)	% Mortality ± SE	LT_{50} (days) (95% FL)
M. anisopliae	ICIPE18	77.5±3.2bcA	2.6 (2.4-2.8)	74.3 ±11.8bAB	3.8 (3.7-4.0)	61.9±4.5bcB	4.7 (4.5-4.8)
	ICIPE30	98.3±1.7aA	2.9 (2.8-3.0)	96.8±3.2aA	3.4 (3.3-3.6)	72.6±6.0aB	3.7 (3.6-3.9)
	ICIPE62	85.3±2.2abB	1.6 (1.5-1.7)	92.3±3.5abA	1.5 (1.4-1.6)	77.0±7.8abB	2.2 (2.0-2.3)
	ICIPE69	86.6±2.0abA	2.2 (2.1-2.3)	82.6±13.8abAB	3.3 (3.2-3.5)	74.1±5.8abB	2.9 (2.7-3.0)
	ICIPE78	72.0±6.5bcB	2.3 (2.1-2.5)	95.5±3.0aA	2.2 (2.1-2.3)	66.5±8.1bcB	4.5 (4.3-4.8)
B. bassiana	ICIPE10	68.5±6.8cdA	4.6 (4.4-4.7)	64.3±6.7bA	4.2 (4.1-4.4)	57.4±1.8cdB	4.3 (4.1-4.4)
	ICIPE273	52.0±1.4dB	3.8 (3.5-4.2)	61.0±4.9bA	3.3 (3.1-3.5)	57.3±1.9dAB	3.8 (3.6-4.0)
	ICIPE279	75.9±0.3bcA	4.1 (3.9-4.2)	32.8±3.8cB	-	77.1±7.7bcA	1.9 (1.8-2.0)

Means within a row followed by the same lower case letters and upper case letters within column do not differ significantly by Tukey's HSD multiple range test ($P = 0.05$). Means were arcsine square-root transformed before analysis, but values presented the actual percentage mortality. LT_{50} (in days) ± 95% fiducial limit (FL). The LT_{50} value for isolate ICIPE 279 was not computed against *L. pseudobrassicae* since mortality was less than 50% at 7 days post treatment.

4.3.2. Dose-mortality response

Among the 3 isolates (ICIPE 30, ICIPE 62 and ICIPE 69) tested for lethal concentration using 50% mortality, isolate ICIPE 62 had the lowest LC_{50} values of 0.1×10^5, 0.05×10^5 and 0.3×10^5 conidia ml^{-1} on *B. brassicae, L. pseudobrassicae* and *A. gossypii*, respectively (Table 5). Isolate ICIPE 69 had the largest LC_{50} value of 5.64×10^6 conidia ml^{-1} on *A. gossypii* and ICIPE 30 (i.e., 3.01×10^6 conidia ml^{-1}) on *L. pseudobrassicae.* Moreover, slopes of the probit regressions ranged between 2.9 and 9.9.

Table 5. LC_{50} values for selected isolates of *M. anisopliae* against apterous adult aphid pests at 7 days post treatment.

Aphid species	*M. anisopliae* Isolates	LC_{50} ($\times 10^5$conidia ml^{-1}) (95% FL)[a]	Slope[b] ± SE
B. brassicae	ICIPE 30	4.4 (2.9-6.9)	5.3±1.2
	ICIPE 62	0.1 (0.08-0.2)	6.2±1.2
	ICIPE 69	3.6 (2.3-5.8)	4.4±1.1
L. pseudobrassicae	ICIPE 30	30.1 (19-50.5)	5.4±1.2
	ICIPE 62	0.05 (0.02-0.08)	9.9±1.3
	ICIPE 69	2.0 (1.4-2.9)	8.3±1.2
A. gossypii	ICIPE 30	7.7 (5.3-11.0)	7.2±1.1
	ICIPE 62	0.3 (0.1-0.7)	2.9±1.1
	ICIPE 69	56.4 (37.4-89.5)	6.3±1.1

[a]Values in the bracket represent 95% Fiducial Limits.

[b]Non-parallel probit models ± standard error (SE) of the mean.

4.3.3 Conidial production

The conidial production significantly varied according to isolates*insect species ($\chi^2 = 35.13$, df = 4; $P < 0.0001$), isolates*days ($\chi^2 = 31.65$, df = 4; $P < 0.0001$) and isolates*insect species*days ($\chi^2 = 85.69$, df = 8; $P < 0.0001$) (Fig. 9 A, B and C).

Across aphid species and fungal isolates, conidial production increased with increasing number of days. For example, the conidial production ranged from 4.9×10^5 - 3.9×10^6 conidia ml^{-1} on day 3, 3.2×10^6 - 1.3×10^7 conidia ml^{-1} on day 6 and 8.8×10^6 - 1.2×10^7 conidia ml^{-1} on day 9 post-treatment (Fig. 9 A, B and C). On day 6 post-treatment, ICIPE 62 produced significantly higher number of conidia across aphid species compared to the other isolates. On the day 9 post-infection, ICIPE 62 outperfomed the other isolates, except ICIPE 30 that produced higher conidia (8.8×10^6 conidia ml^{-1}) on *A. gossypii* than the other isolates (Fig. 9 A, B and C).

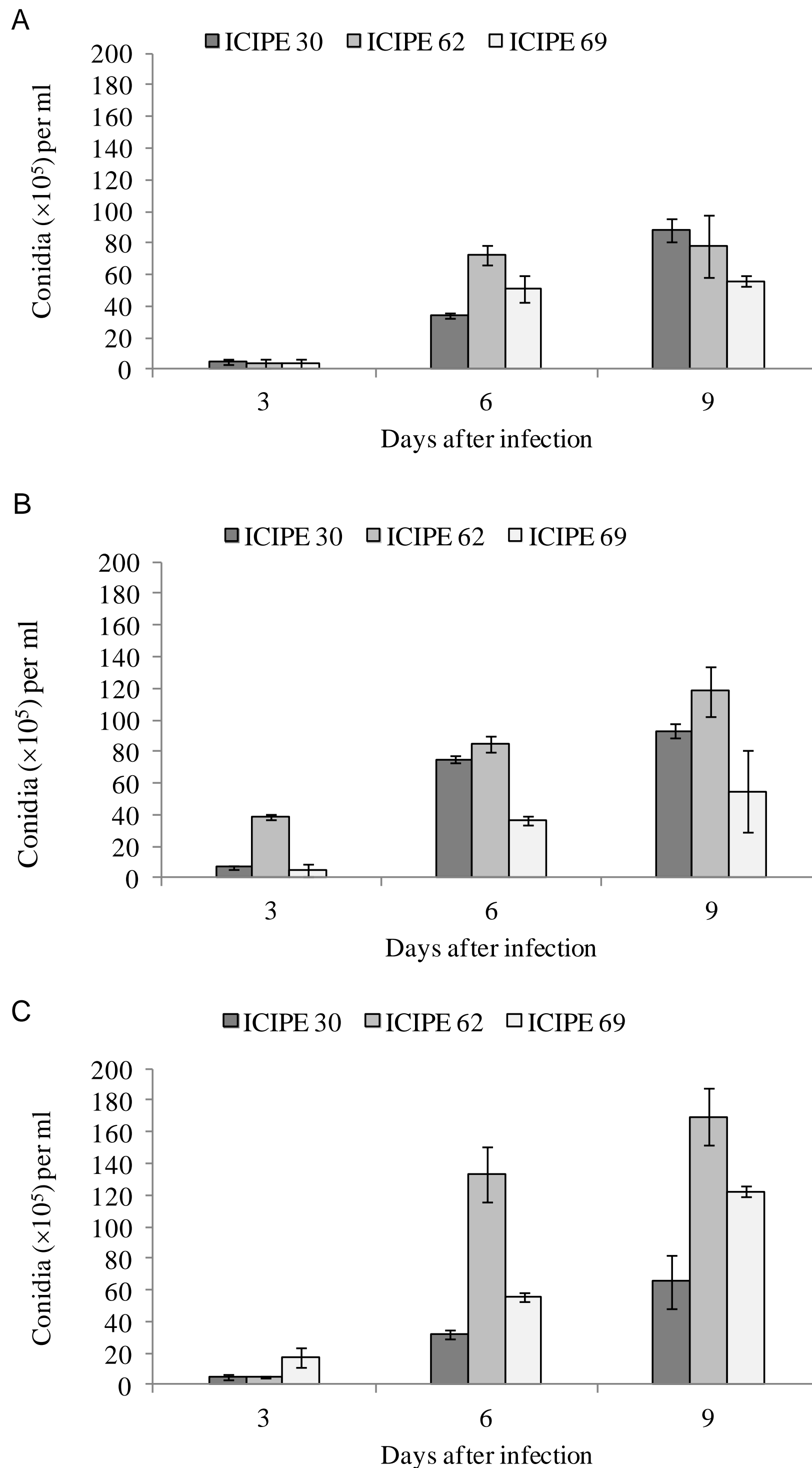

Figure 9. Conidial production on three apterous adult aphids species. (A) *Aphis gossypii*, (B) *Brevicoryne brassicae* and (C) *Lipaphis pseudobrassicae* treated using 1×10^8 conidia ml^{-1} of three isolates of *Metarhizium anisopliae* at 3, 6 and 9 days after treatment. Data present are mean ± SE and were tested at $P < 0.05$. Error bars indicate the standard error (SE).

4.4. Discussion

All the fungal isolates tested were pathogenic to *B. brassicae*, *L. pseudobrassicae* and *A. gossypii.* However, *M. anisopliae* isolates ICIPE 30, ICIPE 62 and ICIPE 69 were more virulent to *B. brassicae* and *L. pseudobrassicae* as compared to *A. gossypii*. Isolates of *B. bassiana* were the least virulent against the three aphid species, except isolate ICIPE 279 which was virulent against *A. gossypii*. These results confirm the pathogenicity of *B. bassiana* and *M. anisopliae* toward aphids as reported by other workers. For example, 100% mortality was reported in third-instar nymphs of *M. persicae* and *A. gossypii* by *B. bassiana* and *M. anisopliae* (de Loureiro and Moino, 2006). Similarly, Jandricic *et al.* (2014) identified a number of isolates of *Beauveria* and *Metarhizium* virulent to first-instar nymphs of *A. gossypii* and *M. persicae* following the screening of 44 fungal isolates and 4 commercially available strains.

Variation in the virulence of fungal isolates has been reported on many groups of arthropods, such as fruit flies (Dimbi *et al.*, 2003), thrips (Ekesi *et al.*, 1998; Niassy *et al.*, 2012), two-spotted spider mite (Bugeme *et al.*, 2009), red spider mite (Wekesa *et al.*, 2005) and leafminer (Migiro *et al.*, 2010). For example, *M. anisopliae* ICIPE 62 which has been found to be virulent against the three species of aphids in the present study, was also reported to be virulent against *Ceratitis rosa* var. *fasciventris* (Karsch), and *C. cosyra* (Walker) (Diptera: Tephritidae) (Dimbi *et al.* 2003) and adult *Cylas puncticollis* Boheman (Coleoptera: Curculionidae) (Ondiaka *et al.*, 2008) but less pathogenic to adult *Ceratitis capitata* (Weidemann) and *Tetranychus evansi* Baker and Pritchard (Acari: Tetranychidae) (Bugeme *et al.*, 2008).

Given the fast developmental period of aphids and the rather short window of opportunity for infection due to ecdysis, the speed of kill becomes essential. In addition to causing high mortalities, *M. anisopliae* isolates ICIPE 30, ICIPE 62 and ICIPE 69 had the shortest LT_{50} values compared to the other isolates against the three aphid species tested. The LC_{50} values varied with aphid species, with *A. gossypii* generally having the highest values, which is an indication that this species is less susceptible to fungal infection by isolates tested. Moreover, the

steepness slopes of probit regressions indicating a moderate to large infection of aphids with conidial concentrations of the fungal isolates.

The identification of fungal isolates with proven efficacy against a broad range of aphid species should be a logical approach for the management of aphid on diverse crops, considering that most of them occupy the same ecological niche than strict specificity to one species. This assertion is in agreement with Jandricic *et al.* (2014) who stated that the ideal myco-insecticide for aphid management should be more consistent, with broader activity against a variety of aphid species than the existing products that are relatively targeted at one species. In this regard, the three *M. anisopliae* isolates (ICIPE 30, ICIPE 62 and ICIPE 69) are potential candidates for development as biopesticides againt the target aphids species compared to the other isolates tested in this study. In addition to the high virulence, ICIPE 62 was proven to produce high number of conidia on the three aphid species.

4.5. Conclusions

This study has identified *M. anisopliae* isolate ICIPE 30, ICIPE 62 and ICIPE 69 as potential candidates for further development as biopesticide for the control of the three aphid species based on the following desirable traits: virulence to the three target aphid pests, conidial production, speed of kill (LT_{50}) and lower LC_{50} values (Yeo *et al.*, 2003). In addition, since aphids usually form large colonies on plant surfaces, the isolate of *M. anisopliae* ICIPE 62 profusely sporulated on dead aphids and could serve as source of inoculum for infection of healthy aphids which may lead to acute mortality. However, the effect of environmental factors, including temperatures on their efficacy need to be assessed to predict their efficacy under field conditions.

CHAPER FIVE: EFFECT OF TEMPERATURE ON CONIDIA GERMINATION, RADIAL GROWTH AND VIRULENCE OF SELECTED *METARHIZIUM ANISOPLIAE* ISOLATES AGAINST APHIDS ON CRUCIFER AND OKRA VEGETABLES

5.1. Introduction

Metarhizium is one of the most commercially important EPF and some have been used as biological control agents against a wide range of arthropod pests (Zimmermann 1993). In the initial experiments, three isolates of *M. anisopliae* showed high virulence to the three target aphid species namely: *A. gossypii, B. brassicae* and *L. pseudobrassicae* (Chapter 3). However, the efficacy of EPF is influenced by a number of factors including abiotic factors (Halla and Papieroka, 1982; Benz, 1987; Inglis *et al.*, 2001).

Temperature is considered as an important factor affecting the efficacy of EPF (Benz, 1987; Maniania and Fargues ,1992; Ekesi *et al.*,1999; Inglis *et al.*, 2001; Dimbi *et al.*, 2004). Although some EPF have a wide range of temperature tolerances, optimum temperature required for growth and infection are usually restricted (Goettel *et al.*, 2000). Temperature dependent growth and infectivity of some EPF has been reported by many workers (Fargues *et al.*, 1992; Vestergaard *et al.*, 1995; Ekesi *et al.*,1999). Apart from the direct effect, the thermoregulation mechanism used by host species can also affect efficacy of EPF. For example, ‘behavioral fever’ is one of the mechanisms that allow the host to counter disease development by raising their internal body temperature (Roy *et al.*, 2006). On the other hand, temperature can prolong or shorten the developmental cycle of insects thereby influencing the infection process of EPF (Fargues, 1972; Fargues and Remaudière, 1977). Understanding of the relationship between temperature and fungal activity is important during selection of isolates for biological control.

The demand for the use of insect pathogens as an alternative pest management necessitates the selection of *M. anisopliae* isolates over wider range of temperatures to predict field efficacy. The objective of this study was, therefore, to assess the effect of temperature on conidial germination, mycelial growth and

virulence of *M. anisopliae* isolates with the goal of selecting isolate that is effective against the three target aphid species at a broad range of temperatures.

5.2. Materials and Methods

5.2.1. Effect of temperature on conidia germination of selected fungal isolates

The three most virulent isolates of *M. anisopliae* isolates ICIPE 30, ICIPE 62 and ICIPE 69 were further evaluated for the effect of temperature on conidial germination. Conidia were harvested and concentration of 3×10^6 conidia ml^{-1} was prepared as described in section 4.2.2. Conidial suspension (0.1ml) was spread-plated on SDA plates and sterile microscopic cover slips placed on each plate. The plates were sealed with Parafilm membrane and later incubated at 10, 15, 20, 25, 30, and 35°C in complete darkness. After 18 h post-inoculation, conidial germination was halted using 1 ml formaldehyde (0.5%) and the plates were assessed for germination as described in section 4.2.2. The experiment was replicated four times for each temperature and fungal isolate.

5.2.2. Effect of temperature on radial growth of fungal cultures

To assess the effect of temperature on radial growth, spore suspension (0.1ml) of isolates ICIPE 30, 62 and 69 titrated to 3×10^6 conidia ml^{-1} was spread-plated on SDA plates and allowed to grow for 3 days to obtain mycelial mats. Plugs (ca. 5 mm) of mycelium were cut from the plates using an 8-mm diameter sterile cork borer and placed upside down at the centre of a 90-mm Petri dish containing sterile SDA. The plates were sealed with Parafilm membrane and incubated for 12 days under the conditions described in section 5.2.1. Four replicates were used for each isolate and temperature combination. The radial growth was measured every other day using two cardinal diameters drawn on the bottom of each plate (Fig. 10).

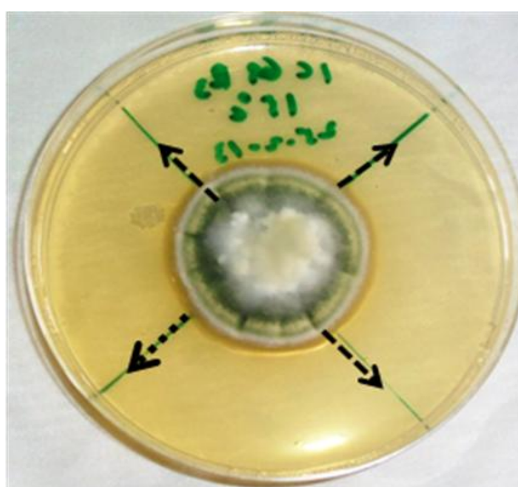

Figure 10. Fungal culture on Saboraud Dextrose Agar (SDA) medium used for radial growth study. Arrow denotes the direction of growth.
Photo: W.H. Bayissa.

5.2.3. Effect of temperature on virulence of entomopathogenic fungi

Thirty apterous adult aphids of each species were treated with 10 ml of conidial suspension titrated 1×10^8 conidia ml^{-1} using Burgerjon spray tower and insects were handled as described earlier. *Aphis gossypii* and *L. pseudobrassicae* were incubated at 15, 20, 25, 30 and 35°C while *B. brassicae* was incubated at 10, 15, 20, 25 and 30°C since it is largely a highland pest. All the treatment combinations were arranged in a complete randomized design with four replicates. Mortality was recorded daily for 5 days. Dead insects were placed on Petri dish lined with moist filter paper as described in section 4.2.3. Mycosis was confirmed by examining the surface of the cadaver under a microscope.

5.2.4. Statistical analysis

All data analyses were done using R v2.14.2 statistical software package (R Development Core Team, 2011). Data on percent mortality were corrected for control values using Abbot's formula (Abbot, 1925) and then normalized by arcsine square-root transformation (Gomez and Gomez, 1984) before analysis of variance (ANOVA). Linear model (y = vt+b) was used to evaluate the influence of

temperature on growth of isolates since the growth after 3-12 days showed linear relationship with the experimental period (Fargues *et al.* 1992). Tukey HSD test was utilized for mean separation. Lethal time to 50% mortality (LT_{50}) was estimated by probit analysis. LT_{50} values were compared by lack of overlap in confidence limits.

5.3. Results

5.3.1. Effect of temperature on conidial germination

Conidia of the three isolates of *M. anisopliae* germinated at all the other temperatures tested (ranging from 1.5-100%), except at 10°C where no germination was observed after 18 h post-incubation (Fig. 11). Significant difference was observed in germination among the isolates at 15°C (F= 21.64; df = 2, 9; P = 0.0004) and 35°C (F = 92.16; df = 2, 9; P < 0.0001). However, there was no significant difference among the fungal isolates at 20°C (F = 0.02; df = 2, 9; P = 0.9809), 25°C (F = 1.18; df = 2, 9; P = 0.3504), and 30°C (F = 1.39; df = 2, 9; P = 0.2972). The interaction between temperature and isolates were also significant (F = 63.74; df = 10, 54; P < 0.0001). The temperature range for optimal conidial germination were observed at 20, 25 and 30°C for all the three isolates (Fig.11).

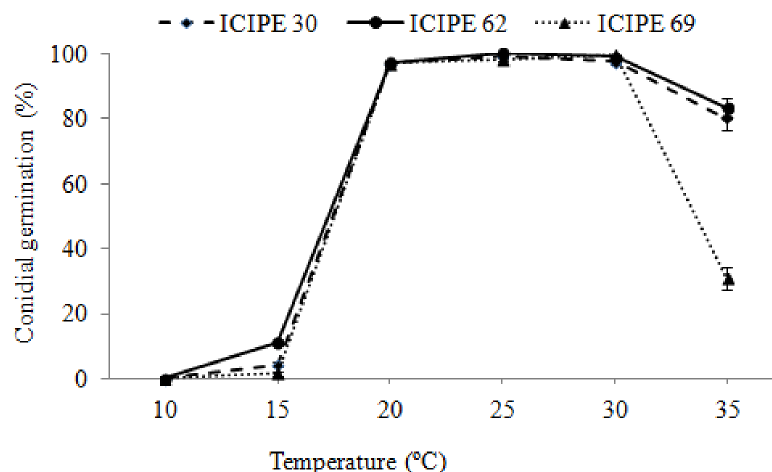

Figure 11. Effect of temperature on conidia germination of selected Metarhizium anisopliae isolates. Bars indicate SE at 95% CI.

5.3.2. Effect of temperature on fungal growth

There were significant differences in growth among the three fungal isolates at 15°C ($F = 16.09$; $df = 2, 9$; $P = 0.0011$), 20°C ($F = 55.37$; $df = 2, 9$; $P < 0.0001$), 25°C ($F = 74.87$; $df = 2, 9$; $P < 0.0001$), 30°C ($F = 102.64$; $df = 2, 9$; $P < 0.0001$) and 35°C ($F = 22.49$; $df = 2, 9$; $P = 0.0003$). Growth was not significantly different among the isolates at 10°C ($F = 3.20$; $df = 2, 9$; $P = 0.0894$) (Table 6).

Table 6. Effect of temperature on growth rate day^{-1} of selected isolates of *M. anisopliae* cultured on SDA medium.

Isolate	Mean growth rate (mm day $^{-1}$) ± SE					
	10°C	15°C	20°C	25°C	30°C	35°C
ICIPE30	0.47±0.02aD	1.06±0.03bC	2.03±0.11cB	4.59±0.13cA	4.46±0.20cA	1.01±0.11cC
ICIPE62	0.47±0.02aE	1.44±0.05aD	4.30±0.14aC	8.39±0.31aA	7.54±0.18aB	1.86±0.06aD
ICIPE69	0.58±0.05aE	1.40±0.11aD	3.15±0.17bC	5.98±0.13bA	5.37±0.16bB	1.09±0.06bD

Means within a column followed by the same lower case letters and within a row followed by the same upper case letters are not significantly different by Tukey's HSD multiple range test (P = 0. 05).

5.3.3. Effect of temperature on virulence of *M. anisopliae* isolates to aphid species

Mortality of apterous adult *B. brassicae, L. pseudobrassicae* and *A. gossypii* due to the three isolates of *M. anisopliae* at various constant temperatures is presented in Table 7. Apterous adult *B. brassicae* were unable to survive at 35°C and was omitted from the analysis.

Overall, the mortality of the three apterous adult aphid species was significantly affected by temperature ($F = 136.46$; df = 4, 121; $P < 0.0001$), fungal isolates ($F = 8.55$; df = 2, 121; $P = 0.0003$) and aphid species ($F = 38.10$; df = 2, 121; $P < 0.0001$). Significant interaction between fungal isolate*temperature ($F = 2.04$; df = 8, 121; $P = 0.0466$), isolate*aphid species ($F = 3.59$; df = 4, 121; $P = 0.0084$), temperature*aphid species ($F = 5.27$; df = 7, 121; $P < 0.0001$), and isolate*temperature* aphid species ($F = 2.85$; df = 14, 121; $P = 0.001$) were also observed.

Across aphid species and fungal isolates, optimum temperature for virulence was found to be 25°C (54.4-91.8%) and 30°C (73.4-93.7) although isolate ICIPE 69 caused higher mortality (81.7%) of *B. brassicae* at 20°C (Table 7). At 15 and 20°C, the performance of each of the isolates in terms of virulence across aphid species was rather unique in that none of the isolates achieved comparable level of mortality on the test aphids. For example, isolate ICIPE 30 induced 25.1 and 34.5% mortality of *A. gossypii* and *B. brassicae*, respectively, but performed poorly (10.4%) on *L. pseudobrassicae* (Table 7). Similarly, at 20°C, isolate ICIPE 62 caused 69.4% mortality of *B. brassicae* and 63.3% of *L. pseudobrassicae* but was less virulent to *A. gossypii* (25.0%) at the same temperature. However, at the optimum temperature of 25-30°C, the three isolates were largely at par in terms of virulence to all the target aphid species (Table 7).

The LT_{50} values decreased with increasing temperatures up to 30°C. The values were not estimated at 15°C for all the three isolates and at 35°C for ICIPE 30 against *A. gossypii* because mortality was less than 50% (Table 6). Generally, the shortest LT_{50} values were observed at the optimal temperatures of 25-30°C for all the three isolates; however, ICIPE 62 killed faster at 30°C (Table 7).

Table 7. Virulence of selected *M. anisopliae* isolates to apterous adult *B. brassicae, L. pseudobrassicae* and *A. gossypii*: percent mortality (±SE) and LT_{50} values 5 days after exposure to 1× 10^8 conidia ml^{-1} under different constant temperatures.

Isolate	Temperature (°C)	*B. brassicae*		*L. pseudobrassicae*		*A. gossypii*	
		% Mortality ± SE	LT_{50} (day) (95% FL)	% Mortality ± SE	LT_{50} (day) (95% FL)	% Mortality ± SE	LT_{50} (day) (95% FL)
ICIPE 30	15	34.5 ± 7.1cA	_	10.4 ±2.5dB	_	25.1 ±3.5 cAB	_
	20	42.9 ± 3.7cA	_	40.2 ± 8.3cA	_	34.3 ± 9.3cA	_
	25	67.4 ± 7.5bB	3.6 (3.4-3.8)	82.1 ± 3.4aA	3.1 (3.0-3.2)	66.6 ± 2.7abB	3.8 (3.7-4.0)
	30	81.4 ± 3.0aA	3.1 (2.9-3.2)	80.3 ± 5.5aA	3.5 (3.4-3.6)	69.7 ± 4.0aB	3.6 (3.4-3.7)
	35	_	_	62.0 ± 7.4bA	4.3 (4.1-4.5)	36.8 ± 10.2bcB	_
ICIPE 62	15	33.3 ± 6.1cA	_	3.3 ± 2.4dB	_	9.2 ± 7.4dB	_
	20	69.4 ± 2.0bA	3.4 (3.3-3.6)	63.3 ± 11.1bcA	4.0 (3.8-4.2)	25.0 ± 5.3cB	_
	25	91.8 ± 3.9aA	3.1 (3.0-3.2)	77.0 ± 3.8bB	4.1 (3.9-4.2)	65.7 ± 4.6bB	4.2 (4.1-4.4)
	30	89.6 ± 4.3aA	2.4 (2.3-2.5)	89.7 ± 2.1aA	2.5 (2.4-2.6)	87.2 ± 1.9aA	2.7 (2.6-2.8)
	35	_	_	49.4 ± 3.3cA	_	56.3 ± 8.2bA	4.5 (4.3-4.7)
ICIPE 69	15	27.5 ± 8.4cA	_	5.6 ± 9.2fB	_	17.7 ± 2.9cA	_
	20	81.7 ± 3.9bA	3.6 (3.5-3.7)	44.9 ± 6.3eB	_	16.0 ± 0.7cC	_
	25	78.3 ± 7.5bA	2.8 (2.7-3.0)	70.0 ± 5.9bA	3.7 (3.5-3.8)	54.4 ± 3.9bB	4.8 (4.4-5.2)
	30	93.7 ± 2.3aA	2.5 (2.4-2.6)	83.9 ± 3.8aAB	3.1 (3.0-3.2)	73.4 ± 4.7aB	3.1 (3.0-3.2)
	35	-	_	57.3 ± 3.0dA	4.3 (4.1 - 4.5)	60.6 ± 8.8abA	4.4 (4.2-4.6)

Means within a column followed by the same lower case and within a row followed by the same upper case letters are not significantly different by Tukey's HSD multiple range test (P = 0. 05). *Values in the bracket represent 95% Fiducial Limits. Dash (–) means the LT_{50} value was not estimated for cumulative mortality less than 50% at 5 days post treatment, and also there is no data on *B. brassicae* at 35°C.

5.4. Discussion

Conidial germination and mycelial growth of the isolates were observed at all temperatures tested with the optimum temperature of growth occurring at 25 and 30°C, which is in agreement with previous published reports (Fargues *et al.*, 1992; Ekesi *et al.*, 1999; Dimbi *et al.*, 2004; Bugeme *et al.*, 2008; Migiro *et al.*, 2010). The thermotolerance of *M. anisopliae* ICIPE 62 observed in the present study is in agreement with the findings of Dimbi *et al.* (2004). On the other hand, *M. anisopliae* ICIPE 69 was found not to tolerate the temperature of 35°C. Similar observation was reported by Ekesi *et al.* (1999).

Mortality of the three apterous adult aphid species was significantly affected by temperature, fungal isolates and aphid species. However, the optimal temperatures for virulence to the 3 aphid species were 25 and 30°C which corresponded to optimal temperatures for germination and radial growth. While evaluating the effect of temperature on virulence of isolates of *B. bassiana* and *M. anisopliae* to *T. urticae,* Bugeme *et al.* (2009) reported that both fungal species were more virulent at 25, 30 and 35°C than at 20°C. A correlation between optimum temperature for fungal growth and fungal infection has been reported by different authors (Maniania and Fargues, 1992; Ekesi *et al.*, 1999; Bugeme *et al.*, 2008).

5.5. Conclusions

This study has generated more knowledge on the adaptation potential of *M. anisopliae* isolate ICIPE 62 to a broad range of temperature compounded with high virulence against target pests. This knowledge is crucial to predict activity of the isolate at different ecologies for consideration in biological control. Moreover, thermotolerance of isolate ICIPE 62 to broad temperature range compares favourably with prevailing temperatures under which the three aphid species thrive. Isolate ICIPE 62 was therefore selected for further evaluation in screenhouse and for compatibility studies with other control agents such as predators and parasitoids.

CHAPTER SIX: INTERACTIONS AMONG VEGETABLE-INFESTING APHIDS, THE FUNGAL PATHOGEN *METARHIZIUM ANISOPLIAE* (ASCOMYCOTA: HYPOCREALES) AND THE PREDATORY BEETLE *CHEILOMENES LUNATA* (COLEOPTERA: COCCINELLIDAE)

6.1. Introduction

Previous chapters have demonstrated the pathogenicity of five isolates of *M. anisopliae* and three of *B. bassiana* against three vegetable infesting aphid species namely: *A. gossypii, B. brassicae* and *L. pseudobrassica*e. Of all the isolates, *M. anisopliae* ICIPE 62 is highly pathogenic to the three aphid species causing mortality of 83-97%; high conidial yield and broad thermotolerance. Isolate ICIPE62 also had the shortest LT_{50} values of 2.8, 2.1 and 1.9 d; and the lowest LC_{50} values of 5.5×10^5, 8.1×10^4 and 1.7×10^4 conidia ml^{-1} against *A. gossypii, B. brassicae* and *L. pseudobrassicae*, respectively (Chapter 4 and 5).

Aphids are attacked by various arthropod natural enemies including predators such as coccinellids which are widely recognized in the suppression of aphid populations (Obrycki and Kring, 1998, Schellhorn and Andow, 2005; Van Emden and Harrington, 2007; Evans, 2009; Giorgi *et al*., 2009). For instance, *Cheilomenes lunata* Fabricius (Coleoptera: Coccinellidae) has been documented in many parts of Africa as an important biocontrol agent of different aphids species (Brown, 1972; Ofuya, 1995; Adisu and Freier, 2003; Woin *et al*., 2006). In Kenya, it has been observed preying on *B. brassicae* and *A. gossypii* on crucifers and okra plants (S. Ekesi, unpublished).

Despite their role as a biocontrol agent for aphids, coccinellids are also known to be susceptible to EPF such as *Hesperomyces virescens* Thaxter and *B. bassiana.* In fact *B. bassiana* is widely documented as major mortality factors of overwintering coccinellids (Iperti, 1966; Ceryngier and Hodek, 1996; Barron and Wilson, 1998; Ormond *et al*., 2006) but there are

no similar records for other Hypocreales. As biopesticides and natural enemies (parasitoids and predators) become available and attractive for incorporation into IPM, it is important to understand their interactive roles. Roy and Cottrell (2008) opined that there is considerably less information on interaction between coccinellids and naturally-occurring fungal pathogens and recommended the need for future research to address the profound absence of knowledge.

Several studies have demonstrated the existence of asymmetric and symmetric intra-guild predation between entomopathogens and insects (Roy and Pell, 2000). For example, *C. septempunctata*, *Coccinella septempunctata brucki* Mulsant (Coleoptera: Coccinellidae) and *H. axyridis* consume aphids at a late stage of *Pandora neoaphidis* (Remaudière and Hennebert) Humber infection (Pell *et al.,* 1997*;* Roy *et al.*, 1998, Roy *et al.*, 2003; Roy *et al.*, 2008). It was concluded that these coccinellids have the potential to negatively impact on *P. neoaphidis* dynamics but in all cases a preference was shown for uninfected aphids even though this preference was only marginal for *H. axyridis* (Roy *et al.*, 2008).

Host and non-host dispersal are important for pathogen transmission within an agroecosystem. Roy, Pell, Clark, and Alderson (1998) demonstrated the impact of coccinellid foraging on *P. neoaphidis* transmission within a plant. Similar studies among the Hypocrelean fungi are needed to improve our understanding of insect-host-pathogen dynamics (Roy and Cottrell, 2008).

Metarhizium anisopliae is considered to have minimal risks to non-target organisms (Zimmermann, 2007). However, since it has been reported on more than 200 insect hosts (Veen, 1968), there is need to assess interaction and compatibility with non-target organism if they have to be used together within the context of aphid IPM programs (Jaronski *et al*., 1998). Roy and Cottrell (2008) stated that the bulk of research on the direct interactions between fungi and coccinellids has concentrated on entomophthorales and *B. bassiana* and there was the need for considerably more data on interaction studies among other species. This study was carried out to assess

the pathogenicity of *M. anisopliae* isolate ICIPE 62 against adult *C. lunata,* to evaluate the effects of fungal infection on the predation behavior of *C. lunata* on *M. anisopliae*-infected *A. gossypii, B. brassicae* and *L. pseudobrassicae,* and to assess the likelihood of *C. lunta* to vector fungal conidia to susceptible aphids during foraging on host plants.

6.2. Materials and Methods

6.2.1. Aphid rearing

The stock cultures of aphids (*B. brassicae, L. pseudobrassicae* and *A. gossypii)* originated from field populations infesting cabbage, kale and okra in vegetable production sites of Nyeri County, Kenya (0°21'10.69"S 37°5'14.35"E, 1878 meters above sea level (m.a.s.l)), and Nguruman Village, Kenya (1°48'22.1"S 36°03'41.2"E, 746 m.a.s.l). The cabbage aphids, *B. brassicae* and turnip aphids, *L. pseudobrassicae* were reared on kale (*Brassica oleracea* L. var. 1000-headed) and cotton/melon aphids, *A. gossypii* on okra (*Abelmoschus esculentus* (L.) Moench var. Pusa sawani) in the insectary at 27-28°C, photoperiod of 12: 12 L: D and 45-50% RH. In order to obtain insects of uniform aged groups, apterous adults of each species were removed from the insectary colony and introduced on fresh kale or okra leaves in Petri dishes (90 mm) to larviposit for 24 h. The offspring that were produced were reared synchronously until they reached the desired stage and thereafter used for the bioassays.

6.2.2. Coccinellid colony

The initial cohort (n > 50) of *C. lunata* originated from organic-grown kale or okra plants infested with aphids at the *icipe'*s Thomas R. Odhiambo campus, Mbita Point (0°34'S 34°10'E; 1170 m.a.s.l). The insect was identified using the reference collection materials at *icipe* Biosystematic Unit and the National Museums of Kenya. The adult coccinellids were maintained on aphid-infested kale or okra seedlings at *icipe's* insectary in a mosquito-net cage (40 × 40 × 63 cm) at 27-28°C, photoperiod of 12:12 L: D and 45-50% RH. As with other similar studies (Roy *et al.*, 1998),

coccinellids are generally difficult to rear. In our study, attempt was made to establish laboratory colony of *C. lunata,* however there was high mortality of the immature stages of the coccinelids. Therefore, field collected adults of *C. lunata* were used for this study. In the laboratory, the field collected adult female *C. lunata* were kept individually in transparent 90-mm Petri dish with approximately 2 mm ventilation hole on the lid and fed on aphids (*B. brassicae, L. pseudobrassicae* or *A. gossypii*) for at least two weeks prior to being used for the treatments. For uniformity, only females *C. lunata* were used in the study.

6.2.3. Fungal culture

The *M. anisopliae* isolate ICIPE 62 used in this study was obtained from the *icipe* Arthropod Germplasm Centre. It was maintained on SDA at 26 ± 2°C in complete darkness. The initial stock of this isolate originated from soil sample collected at Matete, Kinshasa (D.R. Congo) in 1990. It was selected for its virulence against *B. brassiace, L. pseudobrassicae* and *A. gossypii* and also broad thermotolerance (Chapter 3 and 4). Conidia harvesting, viability test and quantification were done as described in section 4.2.2.

6.2.4. Inoculation of insects

6.2.4.1. Coccinellid

A set of 20 *C. lunata* adults were transferred into Petri dishes (100 diam. × 15 mm height) and chilled on ice for approximately 5 min to immobilize them. They were then sprayed with 10 ml of aqueous suspension of *M. anisopliae* isolate ICIPE 62 at a concentration of 1×10^8 conidia ml^{-1}. Another set of 20 *C. lunata* was sprayed with sterile distilled water containing 0.05% Triton X-100 and served as a control. Test-insects were then transferred separately to transparent plastic box (120 ×140 × 40 mm) lined with moistened Whatman filter paper to maintain humidity for a period of 24 h at 26 ± 2°C. Each dish contained aphids infested kale or okra as a source of food for the coccinellids. After 24 h, coccinellids were transferred

to a mosquito-net cage (180 × 180 × 180 mm) and then fed on healthy aphids throughout the experimental period. Mortality was recorded daily for 10 days post-treatment. Dead insects were kept individually in a Petri dish lined with sterile moistened Whatman filter paper to initiate mycosis. Mortality due to *M. anisopliae* was confirmed through microscopic examination of hyphae and spores on the surface of the cadaver. The experiment was replicated four times.

6.2.4.2. Aphids

Two-day old apterous adult aphids of each species were transferred on to fresh leaf disks (ca. 80 mm diameter) of kale (for *B. brassicae* and *L. pseudobrassicae*) and okra (for *A. gossypii*) in a 90-mm Petri dish. Insects were allowed to settle on the leaves for about 20 min and were then sprayed with 10 ml of aqueous suspension of *M. anisopliae* isolate ICIPE 62 at a concentration of 1×10^8 conidia ml^{-1} using Burgerjon's spray tower (Burgerjon 1956). Leaf discs containing aphids were allowed to dry for five minutes, after which aphids were transferred to fresh unsprayed surface-sterilized leaf disks (ca. 100 mm diameter) in 120 ×140 × 40 mm plastic box lined with moistened filter paper. Test-insects were incubated at 26 ± 2°C. Dead insects were transferred into 90-mm Petri dish lined with sterile moistened filter paper to allow for development of mycosis on the surface of cadavers. Three- and 5-day old non-sporulating and sporulating cadavers, respectively, were used in the experiment on the effect of starvation period and prey type (fungus-infected aphids) on *C. lunata* predation. In the control, insects were sprayed with distilled water containing 0.05% Triton X-100 and served as source of live or dead insects. Non-infected aphids were killed by freezing at -18°C for 24 h.

6.2.5. Effect of starvation period and prey type on *C. lunata* predation

The effect of starvation and prey type on predation of *C. lunata* was investigated in non-choice and choice experiments. *Cheilomenes lunata* adult females were starved for 0, 24 and 48 h and were transferred individually into 90-mm Petri dishes with ventilated lids containing aphids

prey types. In non-choice experiments, individual coccinellids were offered the following prey types: (i) six dead non fungus-infected aphids, (ii) six live non fungus-infected aphids, (iii) six dead non-sporulating aphids and (iv) six sporulating aphids.

In choice experiments, two sets of bioassays were done using the method described by Roy *et al.* (1998). Individual *C. lunata* were offered the following prey types: (i) three sporulating and three dead non fungus-infected aphids and (ii) three dead non-sporulating and three dead non fungus-infected aphids. Each prey item was placed in 90-mm Petri dish lined with moistened filter paper. For the choice assay, prey item (infected and non-infected aphid cadavers) was placed at random position within a dish. The Petri dishes were then covered with ventilated lids to provide aeration for the coccinellids. Each treatment was replicated eight times. All the experiments were done in the laboratory at 27 - 28°C and 45 - 50% relative humidity (RH). The number of prey items entirely consumed was recorded after 60 min and 180 min for non-choice and 60, 120 and 180 min for choice experiments.

6.2.6. Effect of *C. lunata* foraging on transmission of fungal infection to healthy aphids on kale and okra plants

Kale and okra were planted in 10 × 15 × 20 cm plastic pots by direct seeding. When plants were approximately 20 cm high, they were thinned to one plant/pot, and transferred to a mosquito-netting cage (40 × 40 × 63 cm) (Fig. 12). Thereafter, the kale plants were infested with healthy 30 fourth-instar *B. brassicae* or *L. pseudobrassicae,* and the okra was infested with *A. gossypii.* Test-insects were obtained from the insectary under production conditions as previously described. Aphids were allowed to settle on their respective host plants and thereafter, 5-day old profusely sporulating cadaver(s) of the respective aphid species were placed on the plants at the following densities: (i) one cadaver, (ii) five cadavers, (iii) ten cadavers and (iv) no cadaver (control). One adult *C. lunata* per plant was then introduced in to each cage and allowed to forage on the prey. The same setup was used

for the control group except that no beetle was introduced. After 5 h, the coccinellids were removed from each cage and all surviving aphids were monitored daily for mortality for 7 days. Each treatment was replicated four times. Dead aphids were placed individually in a Petri dish lined with moistened filter paper. Aphid cadavers were assessed for mycosis as previously described for coccinellid bioassay.

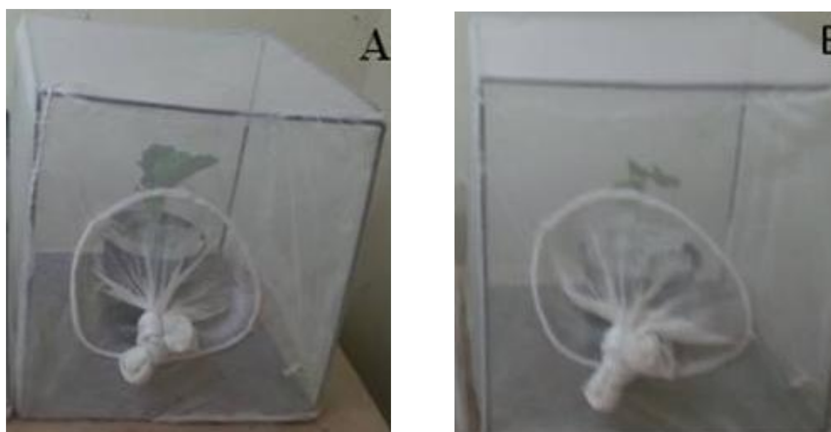

Figure 12. Aphid infested caged host plants (A) kale and (B) okra used in the insectary to study horizontal transmission of EPF by foraging *Cheilomenes lunata*. Photo: W.H. Bayissa.

6.2.7. Data analysis

All data analyses were performed using R v2.14.2 statistical software package (R Development Core Team 2011). Percent mortality data of *C. lunata* was checked for normality and homogeneity of variance using Shapiro-Wilk tests before being subjected to analysis of variance (ANOVA). The data on percentage prey consumed in the non-choice treatments were normalized by arcsine square-root transformation (Gomez and Gomez, 1984) to stabilize the variances before subjecting the data to repeated measures analysis of variance (ANOVA). To assess for the infected and non-infected prey item consumed in the choice treatments, the number of infected (C_i) and non-infected (C_u) prey item were subjected to log transformation after adding 0.05 to allow zero counts (i.e. $r = C_i + 0.05$

and l = C_u+ 0.05) and then log- ratios (i.e. log (l/r) computed: where no preference was shown between prey type then log (l/r) = 0, non-infected aphids preferred prey type, log (l/ r) > 0, and infected aphids were preferred prey type, log (l/ r) < 0 and then repeated measures ANOVA was applied on log-ratio values (Roy *et al*., 2008). Generalized linear model (i.e. binomial error) was used to assess the effect of cadaver density and coccinellid vectoring role on spread of conidia to aphid population.

6.3. Results

6.3.1. Pathogenicity of *M. anisopliae* against *C. lunata*

At the concentration of 1 x 10^8 conidia ml^{-1}, *M. anisopliae* ICIPE 62 caused mortality of 7.5% in *C. lunata* after 10 days post-treatment while mortality in the control was 2.5% and was significantly different ($F = 6$, df =1, 6, P= 0.049) from the fungus treatment (Fig. 13).

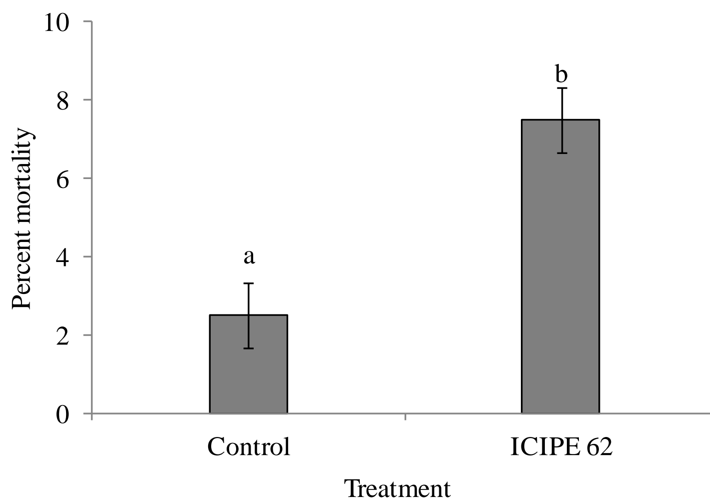

Figure 13. Percent mortality of *Cheilomenes lunata* caused by *M. anisopliae* 10 days post treatment. Bars indicate means SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments Student–Newman–Keuls test at $P = 0.05$.

6.3.2. Effect of starvation period and prey type (fungus-infected aphids) on *C. lunata* predation

6.3.2.1. Non-choice test

Regardless of starvation status and prey species, *C. lunata* did not consume fungus-infected aphids at 60 and 180 min observation periods (Figs. 14 and 15). However, they fed on both live and dead non-infected aphids. Among the non-infected aphids, more *A. gossypii* (35.4-95.8%) were consumed by the predator compared to *B. brassicae* (10.4-60.4%) and *L. pseudobrassicae* (10.4-72.9%) across the observation period.

Predation was significantly influenced by aphid species ($F = 84.48$; df = 2,252; $P < 0.0001$), prey type ($F = 643.45$; df = 3, 252; $P < 0.0001$) and coccinellid starvation ($F=16.77$; df = 2,252; $P < 0.0001$) over a period of 60 and 180 min observation. Significant interaction was also observed between aphid species and prey type ($F = 29.20$; df = 6,252; $P < 0.0001$) and, prey type and coccinellid starvation time ($F = 5.96$; df = 6,252; $P < 0.0001$) in determining prey consumed by coccinellid. For example, consumption of 48 h starved *C. lunata* of dead non-infected *A. gossypii* increased from 70.8 to 83.3%. Overall, consumption of coccinellids increased with increasing starvation period, except for 24 and 48 h starved coccinellids when offered live non-infected *A. gossypii* and dead non-infected *L. pseudobrassicae* after 60 and 180 min, respectively. However, interaction between prey species and coccinellid starvation time ($F = 1.2$; df = 4,252; $P = 0.310$) and among aphid species, prey type and coccinellid starvation ($F =0.98$; df =12,252; $P = 0.465$) had no significant effect on coccinellid consumption over observation periods. Similar trend was also observed when non-infected *B. brassicae* and *L. pseudobrassicae* prey type offered to the coccinellid (Figs. 14 and 15).

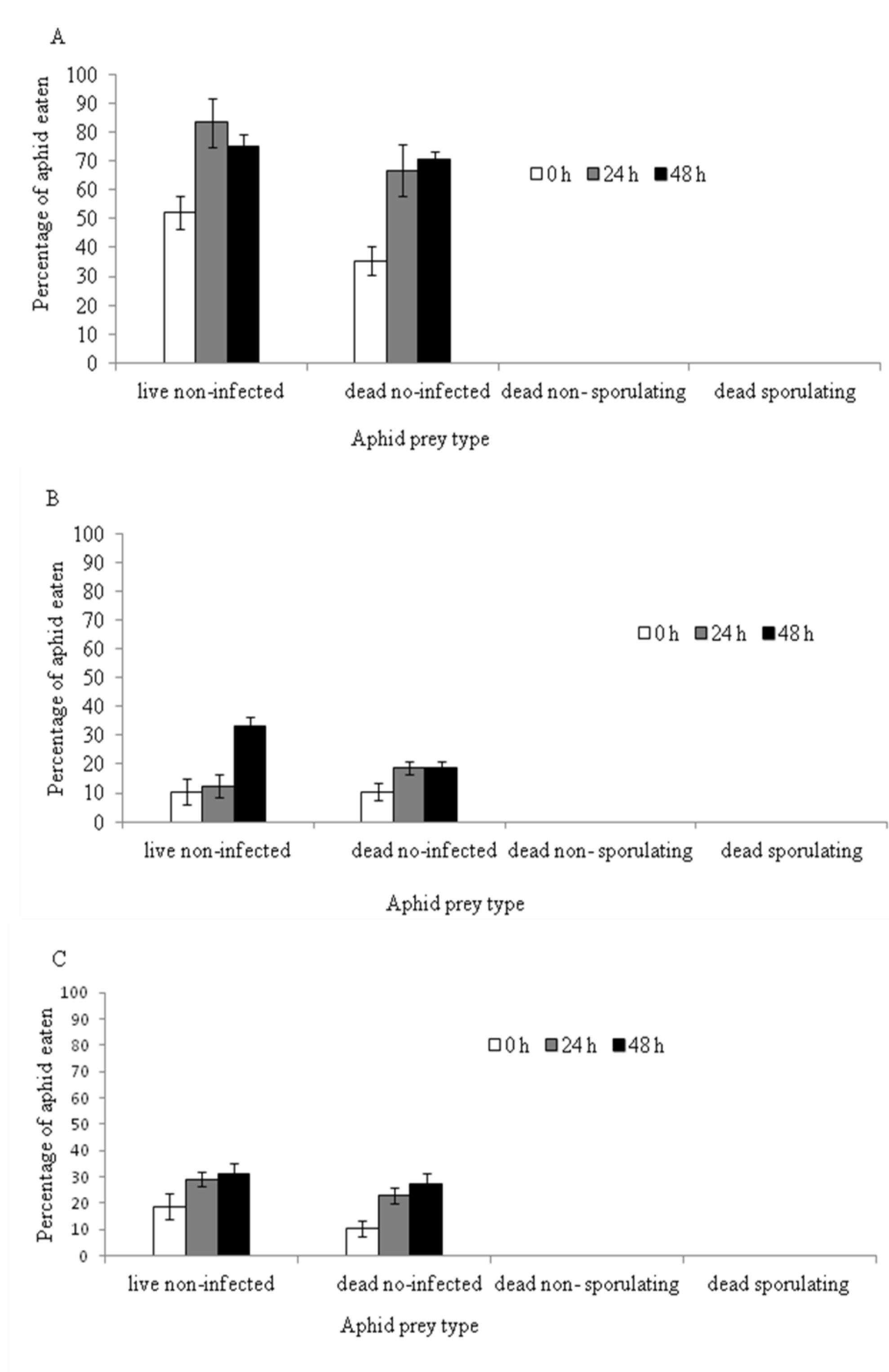

Figure 14. Effect of stage of fungal infection on the proportion of *A. gossypii* (A), *B. brassicae* (B) and *L. pseudobrassicae* (C) eaten by *C. lunata* after 1h.

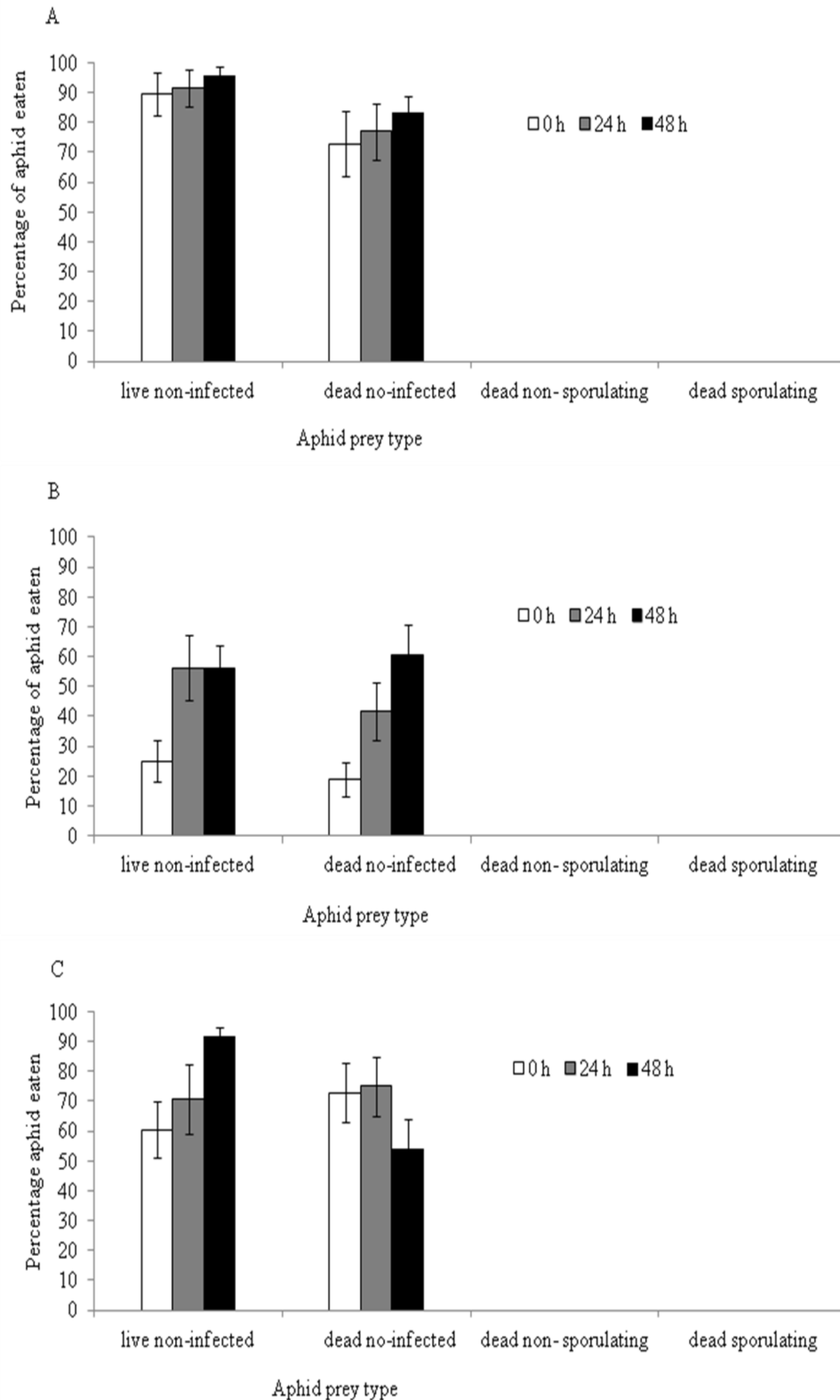

Figure 15. Effect of stage of fungal infection on the proportion of *A.* gossypii (A), *B. brassicae* (B) and *L. pseudobrassicae* (C) eaten by *C. lunata* after 3h.

6.3.2.2. Choice test

In the choice bioassay, the mean numbers of non-infected and fungus-infected (sporulating and non-sporulating) aphid species consumed by coccinellids at different starvation time are presented in Table 8. Starved and nonstarved coccinellids were able to differentiate between sprorulating and non-infected aphid cadavers and showed feeding avoidance behavior to fungus-infected cadavers over the period of observation (Table 8). However, 48 h-starved coccinellids attempted to feed on non-sporulating aphid cadavers. For example, 6.3% of non-sporulating cadavers of *A. gossypii* and 2.1% of *B. brassicae* were consumed during the first 60 min observation time by 48 h-starved coccinellids. In the case of *L. pseudobrassicae,* predation (2.1% of non-sporulating cadavers) by 48 h-starved coccinellids occured after 180 min observation time.

Overall, coccinellid foraging behavior was significantly affected by aphid species (F= 19.24; df = 2,126; $P < 0.0001$) and coccinellid starvation status (F= 8.28; df = 2,126; $P < 0.0004$). For example, 48 h-starved *C. lunata* consumed 83.3, 60.4 and 54.2% of dead non-infected *A. gossypii*, *B. brassicae* and *L. pseudobrassicae* respectively, over 3-h observation periods. Moreover, interaction between aphid species and prey type (F= 7.51; df = 2,126; $P = 0.001$), prey type and coccinellid starvation status (F= 14.79; df = 4,126; $P < 0.0001$) had significant effect on coccinellid foraging behavior over the observed duration of time.

The mean differences between infected and non-infected aphids consumed by coccinellid were greater than zero (Figs 16 A, B and C). These values however, varied with coccinellid starvation status and aphid prey species. For example, the highest values of mean difference (M_D) between fungus-infected and non-infected cadavers is 1.79 and standard error of difference (SE_D) = 0 for *A. gossypii,* and M_D = 1.76, SE_D = 0.02 for *B. brassicae* and M_D = 1.66, SE_D = 0.06 for *L. pseudobrassicae* after 180 min. Overall, the results showed that non-infected prey were preferred by the coccinellid in all the three aphid species (Figs. 16 A, B and C).

Table 8. Mean (±SE) number of *M. anisopliae* infected and non-infected cadavers consumed by *C. lunata* when given as a choice over a period of 180 min.

Aphid species	Coccinellid starvation	Mean number of prey consumed by *C. lunata* within:											
		60 min		120 min		180 min		60 min		120 min		180 min	
		SC	NC	SC	NC	SC	NC	NSC	NC	NSC	NC	NSC	NC
Ag	Non-starved	0.0±0.0	1.6±0.2	0.0±0.0	2.1±0.1	0.0±0.0	2.6±0.2	0.0±0.0	1.9±0.2	0.0±0.0	2.3±0.2	0.0±0.0	2.8±0.2
	24 h starved	0.0±0.0	2.8±0.3	0.0±0.0	3.0±0.0	0.0±0.0	3.0±0	0.0±0.0	1.9±0.2	0.0±0.0	2.3±0.2	0.0±0.0	2.9±0.1
	48 h starved	0.0±0.0	1.0±0.4	0.0±0.0	1.6±0.4	0.0±0.0	1.9±0.4	0.4±0.2	1.8±0.4	0.4±0.2	2.3±0.4	0.4±0.2	2.6±0.2
Bb	Non-starved	0.0±0.0	0.3±0.2	0.0±0.0	0.3±0.1	0.0±0.0	0.4±0.2	0.0±0.0	0.1±0.1	0.0±0.0	0.4±0.3	0.0±0.0	0.9±0.4
	24 h starved	0.0±0.0	0.5±0.2	0.0±0.0	0.9±0.2	0.0±0.0	1.1±0.3	0.0±0.0	2.0±0.3	0.0±0.0	2.1±0.3	0.0±0.0	2.9±0.1
	48 h starved	0.0±0.0	0.6±0.3	0.0±0.0	1.5±0.4	0.0±0.0	1.5±0.4	0.1±0.1	1.8±0.3	0.1±0.1	2.4±0.2	0.1±0.1	2.6±0.2
Lp	Non-starved	0.0±0.0	1.0±0.2	0.0±0.0	1.3±0.1	0.0±0.0	1.6±0.3	0.0±0.0	1.1±0.2	0.0±0.0	2.0±0.4	0.0±0.0	2.4±0.3
	24 h starved	0.0±0.0	1.5±0.3	0.0±0.0	2.0±0.4	0.0±0.0	2.5±0.3	0.0±0.0	0.5±0.3	0.0±0.0	1.8±0.3	0.0±0.0	1.8±0.3
	48 h starved	0.0±0.0	0.8±0.3	0.0±0.0	2.1±0.4	0.0±0.0	2.3±0.3	0.0±0.0	0.6±0.4	0.0±0.0	1.0±0.4	0.1±0.1	2.0±0.2

Ag- *Aphis gossypii*, Bb- *Brevicoryne brassicae*, Lp- *Lipaphis pseudobrassicae*, SC-sporulating cadavers, NC- non-infected cadavers, NSC- non-sporulating cadavers.

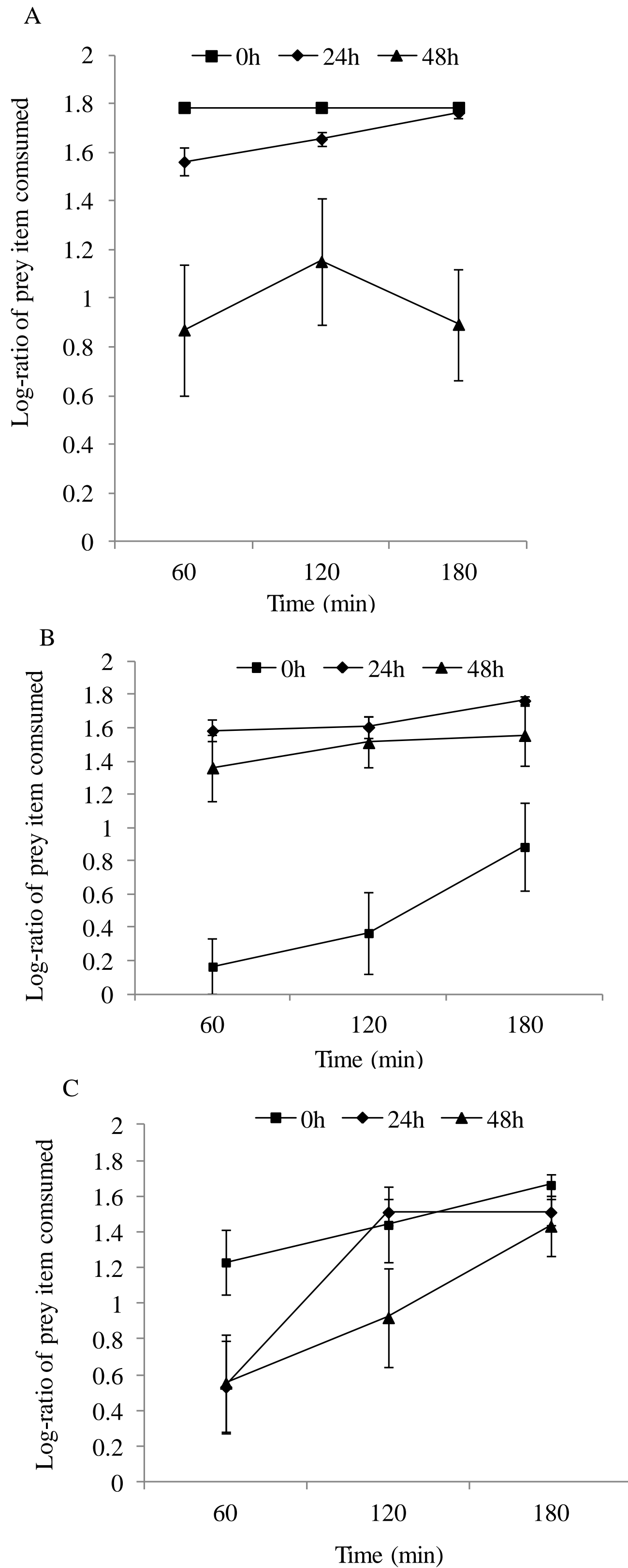

Figure 16. Means of the log-ratios of numbers dead non-infected *A. gossypii* (A), *B. brassicae* (B) and *L. pseudobrassicae* (C) consumed relative to number of infected non-sporulated aphids consumed over a period of 180 min.

6.3.3. Effect of *C. lunata* foraging on transmission of fungal infection to healthy aphids on kale and okra plants

Fungal infection did not occur in all the three aphid species used in the control treatments (absence of fungus-infected cadavers) in the presence or absence of coccinellid, and data were therefore excluded from analysis. Exposure of healthy *B. brassicae, L. pseudobrassicae* and *A. gossypii* on their respective host plants to varying densities of fungus-infected cadavers resulted in fungal infection due to the foraging activity of coccinellids (Fig. 17). However, infection varied according to the density of cadavers, with 10 cadavers having the highest infection rate. In the presence of foraging *C. lunata,* infection significantly increased in *A. gossypii* ($\chi^2 = 3.6$; df = 2; $P < 0.0001$) on okra, and *B. brassicae* ($\chi^2 = 6.4$; df = 2; P = 0.002) and *L. psueudobrassicae* ($\chi^2 = 4.2$; df = 2; $P < 0.0001$) on kale plants compared to the absence of coccinellid (Fig. 17). *Metarhizium anisopliae* profusely sporulated on dead aphids of the three species.

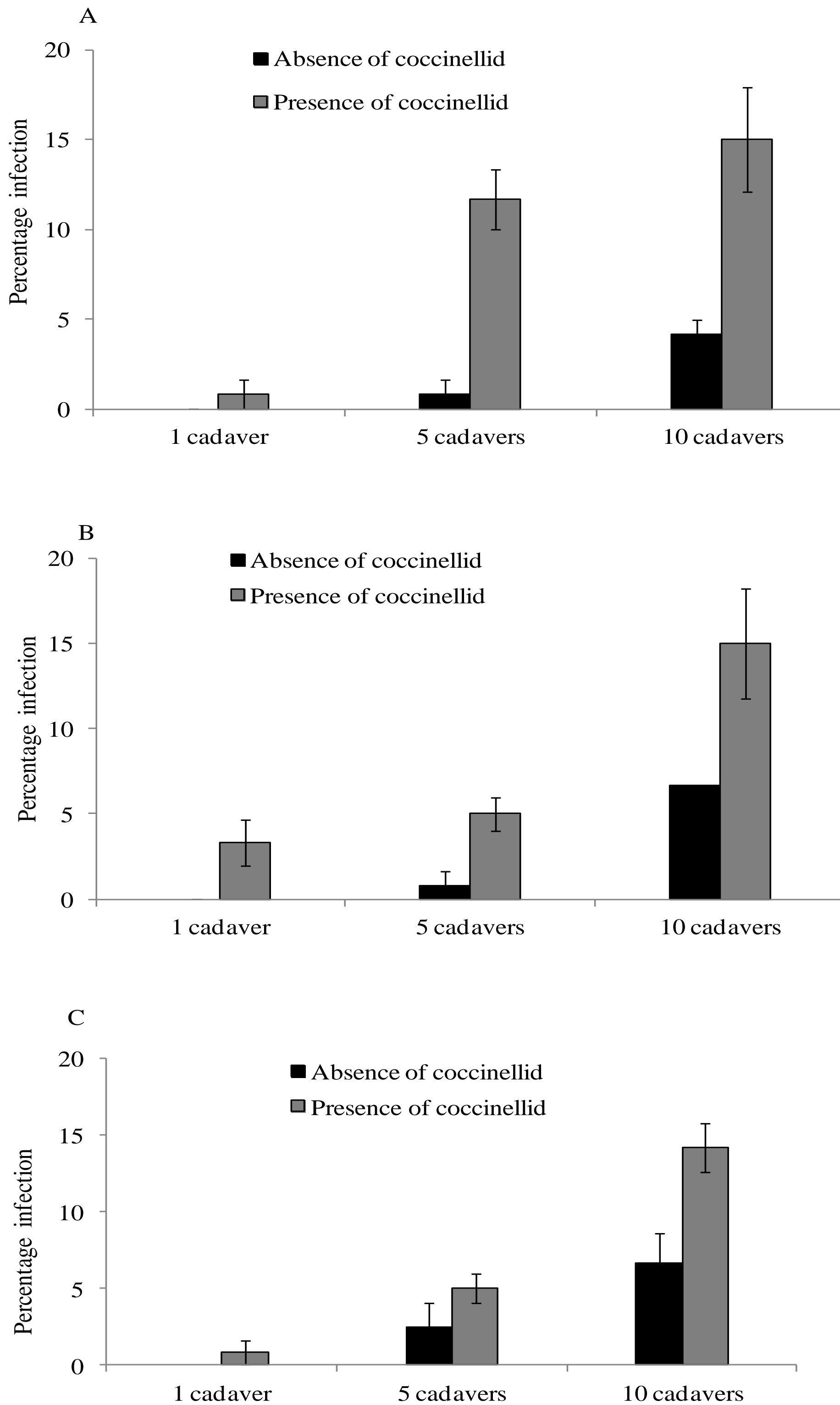

Figure 17. Transmission of *M. anisopliae* ICIPE 62 from cadavers (1, 5 and 10 per plant) to fourth instar *A. gossypii* on okra (A), *B. brassicae* on kale (B) and *L. pseudobrassicae* on kale (C) in the absence or presence of adult female *C. lunata.* Bar represents S.E. at 95% CI.

6.4. Discussion

For successful integration of biological control agents into IPM programs, they must be virulent to the target pests and at the same time have minimal negative effect on nontarget organisms (Goettel *et al*., 1990). *Metarhizium anisopliae* isolate ICIPE 62 whose virulence to the three aphid species namely, *A. gossypii, B. brassicae* and *L. pseudobrassicae,* has been demonstrated earlier (Bayissa *et al.*, 2016) and currently found to be less detrimental to adult *C. lunata* meets these criteria and warrants integration into management programs targeted at the 3 aphid species.

In this study, less than 10% mortality of *C. lunata* was observed after 10 d post-infection with the fungus. Our results support the assertion by various workers that despite the wide host range of *M. anisopliae* , certain strains and genotypes are more restricted to particular species of insects (Bidochka and Small, 2005). Host specificity is indeed one of the key criterion required for selection of some microbial agents for biological control. Furthermore, some isolates of EPF are also more specific under field conditions compared to laboratory studies (Jaronski *et al*., 2003). For example, Ekesi *et al.* (1999) found no adverse effects on nontarget organisms including coccinellids following applications of *M. anisopliae* to control *Megalurothrips sjostedti* (Trybom) in cowpea agroecosystem. A similar result was also reported in onion cropping systems following weekly spray of *M. anisopliae* to control *Thrips tabaci* L. (Maniania *et al.*, 2003). On the contrary, Ginsberg *et al.* (2002) showed that an isolate of *M. anisopliae* was pathogenic to the convergent ladybird beetles, *Hippodamia convergens* Guérin-Méneville in the laboratory. However, in this study, high mortality was observed only over a period of 30 days.

The underlying mechanism for the lack of susceptibility of *C. lunata* to infection by *M. anisopliae* in our study is still unclear. However, the hardened elytra that covers the cuticle constitutes a barrier to conidial attachment, considering that cuticle play a significant role in host susceptibility or resistance to fungal infection (St. Leger *et al*., 1992; Ortiz-Urquiza and Keyhani, 2013).

In non-choice test, our results showed that preying of *C. lunata* on *M. anisopliae*-infected aphids were not affected by the interaction effect of prey species, prey type and coccinellid starvation status within 180 min observation period. This would probably reduce the intraguild interactions between the coccinellid and fungal pathogen. However, when *C. lunata* was offered the choice between non-sporulating and dead non-infected aphid prey, few infected non-sporulating aphid cadavers were perceived as a food source by 48 h starved *C. lunata* during the first 60 min. When offered the choice between sporulating and dead non-infected aphid cadavers, the coccinellids did not feed on sporulating aphid cadavers; which suggests the ability of *C. lunata* to discriminate among different stages of infection of the prey by fungal pathogen as previously observed in *Dicyphus hesperus* Knight (Alma *et al*., 2010). Pell and Vandenberg (2002) demonstrated that the convergent ladybird, *H. convergens*, avoided feeding on Russian wheat aphids, *Diuraphis noxia* (Mordavilko) (Hemiptera: Aphididae), infected with *P. fumosoroseus*. Feeding avoidance of *C. lunata* on fungus-infected cadavers observed in the present study is also similar to the one reported with other generalist insect predators: *Coccinella septempunctata* L. (Pell *et al*., 1997), *Anthocoris nemorum* L. (Meyling and Pell, 2006) and *Orius albidipennis* Reuter (Pourian *et al*., 2011).

In the single caged-plant foraging experiments, the precence of adult *C. lunata* enhanced spread of *M. anisopliae* from infected cadaver and increased infection of *A. gossypii* on okra, and *B. brassicae* and *L. pseudobrassicae* on kale and okra. The high mobility of adult coccinellid (Dixon *et al*., 1997) might have increased the spread of conidia and subsequent contamination of healthy aphids. Moreover, the escape response to avoid predation in aphids would increase acquisition of conidia through direct contact with the cadaver.

The role of *C. lunata* in vectoring inoculum observed in the present study is similar to the one reported with *C. septempunctata* (Pell *et al*. 1997; Roy *et al*., 1998, Ekesi *et al*., 2005). Variation of infection of aphids following exposure to different cadaver density observed in the current study can be explained by high density of conidia received by susceptible host (Vandenburg *et al*., 1998). However, infection was generally low across the three aphid species. The

foraging period of coccinellids has probably an effect on conidial dissemination that initiate infection on aphids although it was not investigated in the current study. As previously suggested by Ekesi *et al.* (2005), extended time of coccinellid foraging on the plants could increase the infection of aphids. Overall, these behavioural activities should increase secondary uptake of spores from the plant surfaces and enhance infection and subsequent suppression of aphid populations in the agroecosystems.

6.5. Conclusions

In conclusion, *C. lunata* is less susceptible to *M. anisopliae* isolate ICIPE 62 at a concentration that caused high mortality in all the three aphid species tested. In addition, prey preference from *M. anisopliae* -infected to non-infected was not observed as a result of coccinellids starvation status and aphids prey species under prey choice and/or non-choice test. Fungus-infected aphids were not preferred as prey, thus, it is unlikely that coccinellids feeding would reduce the amount of inoculum in order to initiate infection in aphid populations. Rather, the presence of *C. lunata* on host plants enhances transmission and spread of conidia which should be advantageous to the overall aphid suppression strategy. However, further field studies are necessary to generate more information on the interaction among fungal pathogen, coccinellids and aphid pests.

CHAPTER SEVEN: INTERACTION AMONG VEGETABLE INFESTING APHIDS (HEMIPTERA: APHIDIDAE), FUNGAL PATHOGEN *METARHIZIUM ANISOPLIAE* (ASCOMYCOTA: HYPOCREALES) AND PARASITIC WASPS (HYMENOPTERA: APHIDIIDAE) FOR THE MANAGEMENT OF THE PEST ON VEGETABLES

7.1. Introduction

Aphids are attacked by various arthropod natural enemies including parasitic wasps. *Diaeretiella rapae* (M'Intosh) (Hymenoptera: Aphidiidae) is reported to be a dominant species among various parasitoids and widely recognized in the suppression of cruciferous vegetable aphids (Pike *et al.*, 1999; Desneux *et al.*, 2006). Similarly, *Aphidius colemani* (Viereck) is one of the most common species attacking several aphid species, and mainly used in the biological control of *A. gossypii* (van Lenteren, 2003).

In some cases, successful examples of using more than one species for biological control of aphid pests have been documented. For example, the use of *A. colemani* and *Hippodamia convergens* Guerin to control field populations of *A. gossypii* is well documented (Bellows and Fisher, 1999). However, the control efficacy might be compromised as a result of intraguild interaction between different species of natural enemies that share a common host resource (Rosenheim *et al.,* 1995).

Ludwig and Oetting (2001) reported antagonistic interaction between *A. colemani* and commercial formulation of *B. bassiana*. On the other hand, avoidance of fungal infected hosts by ovipositing wasps and a degree of host immunity to fungal infection induced by parasitoid larvae within the host have also been reported (Poprawski *et al.*,1992; Fransen and van Lenteren, 1994). Moreover, the effect of time of application of EPF on compatibility between parasitoids and EPF have also been reported by several workers (Mesquita and Lacey, 2001; Rashki *et al.*, 2009; Aqueel and Leather, 2013).

Veen (1968) listed 204 insects naturally infected by *M. anisopliae* which include both pest and beneficial insect species. However, the degree of specificity varies between and within genera, and strains of the species (Goettel *et al.*, 2010). For example, previous study (chapter 5) have demonstrated compatibility between *M. anisopliae* isolate ICIPE 62 and common aphids predatory beetle, *C. lunata;* and antagonistic relation to the three aphid species is also documented. The results indicate that this fungal isolate holds great potential in suppressing the target aphid species and relatively safe to the non-target predator, *C. lunata* (Bayissa *et al.*, 2016). However, there is no information on the compatible use of *M. anisopliae* isolate ICIPE 62 with *A. colemani* and *D. rapae* for the management of the target aphid species.

As biopesticides and natural enemies (parasitoids) become available and attractive for incorporation into integrated pest management programs (IPM), it is important to understand their interactive roles before recommending them to vegetable growers. This study was therefore carried out to assess the interaction between *M. anisopliae* isolate ICIPE 62 and two aphid parasitoids (*D. rapae* and *A. colemani*) with the aim of developing an IPM strategy for the aphid species that incorporate both biocontrol agents.

7.2. Materials and Methods

7.2.1. Aphid cultures

The stock cultures of aphids (*B. brassicae* and *A. gossypii)* were originally collected from field populations infesting cabbage/kale and okra in vegetable production sites of Nyeri County, Kenya (0°21'10.69"S 37°5'14.35"E, 1878 m.a.s.l), and Nguruman, in Kajiado County, Kenya (1°48'22.1"S 36°03'41.2"E, 746 m.a.s.l). The cabbage aphids, *B. brassicae* were reared on kale and and cotton/melon aphids, *A. gossypii* on okra in the insectary at 27-28°C, photoperiod of 12: 12 L: D and 45-50% RH. In order to obtain insects of uniform age groups, apterous adults of each species were removed from the insectary colony and introduced on fresh kale or okra leaves in Petri dishes (90 mm) to larviposit for 24 h and adults were removed thereafter. The newly-born nymphs were then transferred to fresh seedlings and maintained until adult

stage before they could be used for the bioassay. Two days old adults were used for the subsequent bioassay.

7.2.2. Parasitoids

The stock cultures of *D. rapae and A. colemani* used in this study were obtained from the International Centre of Insect Physiology and Ecology (*icipe*) mass rearing facility. In order to obtain uniform aged wasps, kale or okra seedlings were infested with 2-day old apterous adults of *A. gossypii* and/or *B. brassicae* and maintained in a cage (40 × 40 × 63 cm) at 27 - 28°C, 45 - 50% relative humidity (RH) and photoperiod of 12: 12 L: D). Thereafter, mated females of *A. colemani* and/or *D. rapae* (Figs. 18) were released into the cages. Mummified aphids were collected and kept in Perspex cage (15×15×15 cm) in the insectary at conditions mentioned above. Thereafter, emerged wasps were fed on drops of honey (50%) until they could be used for the bioassay.

Figure 18. *Aphidius colemani* (A) female and (B) male; *Diaeretiella rapae* (C) female and (D) male. Photo: W.H. Bayissa.

7.2.3. Fungal culture

The *M. anisopliae* isolate ICIPE 62 used in this study was obtained from the *icipe* Arthropod Germplasm Centre. It was maintained on Sabouraud dextrose agar (SDA) at 26 ± 2°C in complete darkness. This isolate was isolated from soil in Matete, Kinshasa (D.R. Congo) in 1990. It was selected for its virulence and thermotolerance against *B. brassiace, L. pseudobrassicae* and *A. gossypii* (Chapter 4 and 5). Conidia harvesting, viability test and quantification were done as described in section 4.2.2.

7.2.4. Interactions among *M. anisopliae*, parasitoids and host aphids

7.2.4.1. Aphid first exposed to the parasitoid and then treated with the fungus

Batches of 20 apterous adult aphids were placed on fresh kale or okra in transparent plastic dish (120 mm dia). Each batch of aphids was exposed to 2-day-old mated female *A. colemani* or *D. rapae* for 24 h. Parasitoids were removed from the arena and the parasitized aphids were sprayed with conidial concentration that kills 25%, 50% and 75% (LC_{25}, LC_{50} or LC_{75}) of *A. gossypii* and *B. brasscae* (see chapter 4) after 24, 48 or 72 h. The control group was sprayed with sterile distilled water containing 0.05% Triton X-100. Treated aphids were then transferred to fresh leaves in Petri dishes (90 mm diameter) lined with moisten filter paper and incubated at 26 ± 2ºC and photoperiod of L:D 12:12 h. Each treatment was replicated five times. The numbers of dead aphids were recorded daily and assessed for mycosis and mummification 24 h after fungal treatment for 12 days. The mummified aphids were placed individually in eppendorf tube until emergence of the wasps. Pre-emergence time, percent parasitism, sex ratio, longevity of females (F_1 generation) and tibia length and costal vein of female F_1 generation of the wasps were determined. To assess longevity of female F_1 generation, wasps were individually transferred to screened transparent plastic vials (50 × 100 mm) and fed on honey (50%) at 27-28°C, 45-50% RH, and photoperiod of 12:12 L:D. To measure the tibia and costal veins, the wasps were dissected and mounted on a slide. Images of the tibia and costal vein (Fig. 19 A and B) from

the slide mounted specimens were captured using video microscopy-Leica MZ 125 Microscope (Leica Microsystems Switzerland Limited) , fitted with Toshiba 3CCD camera using the Auto Montage software (Syncroscopy, Synoptics group, Cambridge, UK) at magnification X 25. Measurements were taken by the program Image-Pro® Plus version 4.1 for Windows™ (Media Cybernetics, Bethesda, MD, USA).

7.2.4.2. Aphid first treated with fungus and then exposed to parasitoids

Twenty apterous adults of aphids were sprayed with conidial concentration of *M. anisopliae* that kills 25, 50 and 75% of treated aphid population (see section 6.2.4.1). The aphids were then parasitized by a 2-day-old female mated *A. colemani* or *D. rapae* at different post spray time intervals (24, 48, or 72 h). The control groups (i.e. parasitized aphids) were sprayed with 0.05% Triton X-100. As described above, dead aphids were removed daily, surface sterilized and were placed on moistened filter paper to confirm infection by *M. anisopliae*. The number of mummies produced was recorded. The mummified aphids were collected daily and placed individually in Eppendorf tube. Time of pre-emergence, percent emergence, sex ratio, longevity of females F_1 generation, tibia length and costal veins of females F_1 generation was determined as described in section 7.2.4.1. To investigate longevity of female F_1 generation, emerged wasps were individually transferred to screened transparent plastic vials (50 × 100mm) and maintained as described in section 7.2.4.1.

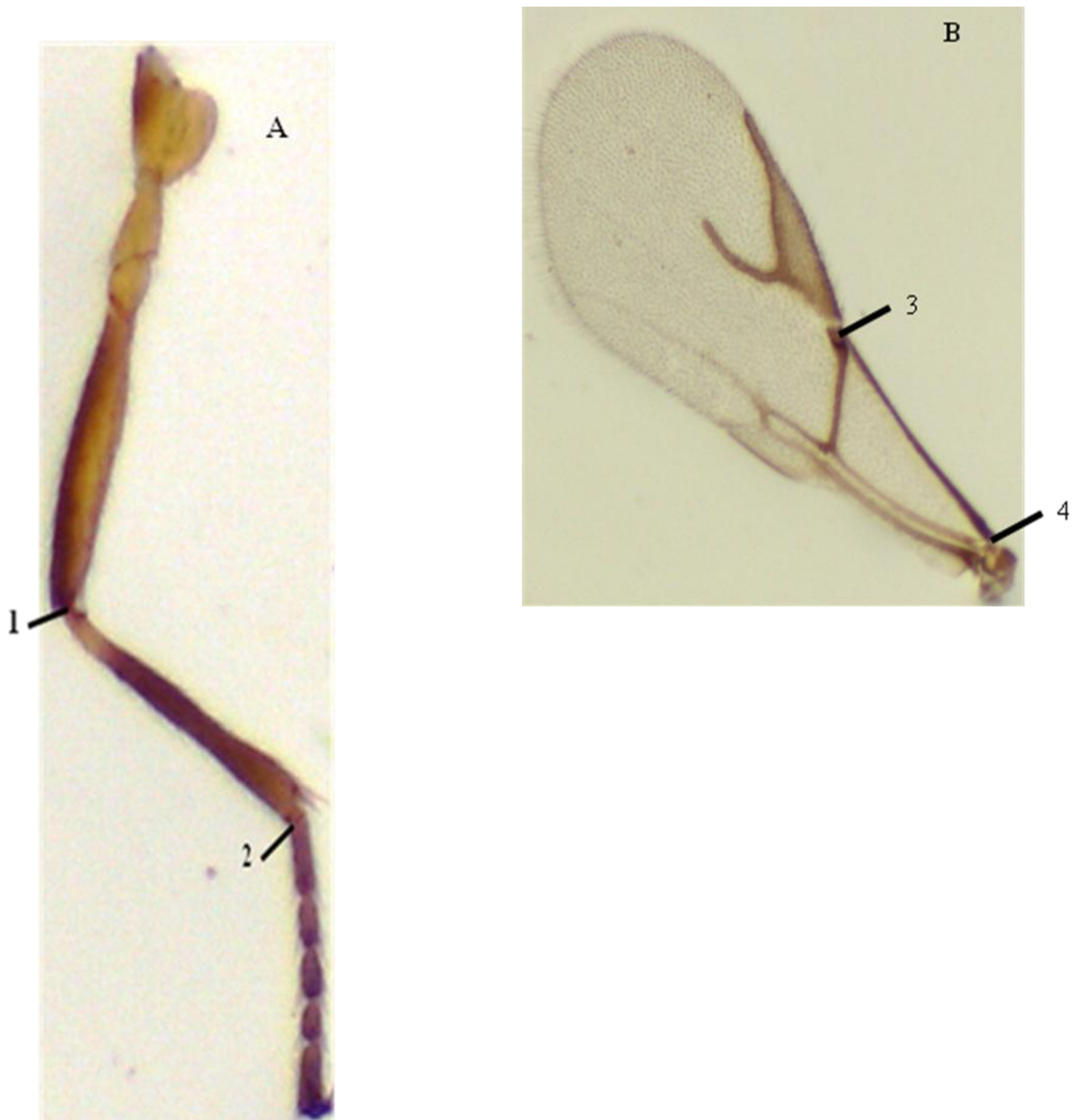

Figure 19. (A) Tibia points of measurement (measurement taken from point 1 to point 2) (B) Costal vein points of measurement (measurement taken from point 3 to point 4). Photo: W.H. Bayissa.

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7.2.5. Statistical analysis

All data analyses were carried out using R v2.14.2 statistical software package (R Development Core Team, 2011). Data on the percentage parasitism, the percentage emergence and female sex ratio of *D. rapae* and *A. colemeni* were analysed separately for both parasitoids species using GLM with binomial errors (or quasi-binomial if data were over-dispersed). The data on development time and longevity of F_1 generation, measurement of tibia and wing vein length of *A. colemani* and *D. rapae* were analyzed using a combination of ANOVA and Student's *t* tests. Student`s *t* tests were used to analyze differences in male and female morphometry. Means were compared with Student-Newman-Keuls test.

7.3. Results

7.3.1. Aphis gossypii first exposed to *A. colemani* and then treated with *M. anisopliae*

There was no significant variation of percent parasitism when *A. gossypii* was first exposed to female *A. colemani* and then treated with conidial concentration (1×10^5, 3.2×10^6 or 1×10^8 conidia ml $^{-1}$) of *M. anisopliae* ICIPE 62 after 24, 48 and 72 h compared to control (P = 0.05). The mean percent parasitism in fungal treated groups varied from 32-74% and 43.5-67.7% in the control (Table 8). Percent parasitism increased with the time interval between parasitoid oviposition and infection by the fungus. For example, 43.0% (95% CI = 31.5-55.5) and 71.0% (95% CI = 58.6-80.9) parasitized *A. gossypii* were observed when first exposed to *A. colemani* and then treated with 1×10^5 conidial ml $^{-1}$ (LC_{25}) after 24 and 72 h, respectively. The variation was statistically significant at $P < 0.05$ (Table 9).

On the other hand, time from oviposition to emergence was not significantly different between fungus treated and control (F = 2.1; df= 9,39; P = 0.053). Similarly, no significant difference between percent emergence of *A. colemani* and between fungus treated and control *A. gosspii* at 24, 48 and 72 h post treatment (P = 0.05). However, variation in percent emergence of *A. colemani*

was observed between aphids treated at LC_{25} and LC_{50} (P = 0.048), and between LC_{25} and LC_{75} (P = 0.031) after 24 h. On the other hand, percent female F_1 generations (F = 1.3; df= 9,37; P = 0.26), longevity of female F_1 generation (F = 0.92; df= 9,79; P = 0.51) and male F_1 generation (F = 0.63; df= 9,82; P = 0.77) were not significantly differ between fungus treated and control groups (Table 9).

The costal wing vein length ranged from 0.68 ± 0.05 to 0.81 ± 0.04 mm for females and 0.62 ± 0.06 to 0.82 ± 0.10 mm for males *A. colemani* F_1 generations that emerged from untreated *A. gossypii* and those that had been parasitized and then treated with different concentrations of conidia of *M. anisopliae* after 24, 48 and 72 h post treatment. There was no significant variation between control and fungus treated groups irrespective of sex (P > 0.05). However, significant variation was observed between male and female *A. colemani* that emerged from *A. gossypii* that had been parasitized and then treated at LC_{25} (t =3.05; P = 0.009) and LC_{50} (t =3.68; P = 0.003) after 24 h, and LC_{75} (t =2.87; P = 0.01) after 72 h. The remaining treatments showed no significant difference (P > 0.05) (Fig. 20).

The length of the metathoracic tibia ranged from 0.47 ± 0.06 to 0.69 ± 0.05 mm for females and 0.41 ± 0.07 to 0.52 ± 0.03 mm for males of *A. colemani* F_1 generations that emerged from parasitized untreated *A. gossypii* and those that had been treated with *M. anisopliae* after 24, 48 and 72 h. No variation was observed between the control and treated groups regardless of sex (P > 0.05). However, significant difference was observed in the tibial length between male and female *A. colemani* that emerged from untreated control (t =9.81; P < 0.001), fungus treated at LC_{50} (t = 5.32; P < 0.001), LC_{50} (t = 2.83; P = 0.012) and LC_{75} (t =2.75; P = 0.013) after 24, 48 and 72 h, respectively (Fig. 21).

Table 9. Effect of *Metarhizium anisopliae* conidia dispensed at varying lethal concentration that kill *A. gossypii* ($LC_{25,}$ LC_{50} and LC_{75}) on parasitism, emergence, developmental time, female F_1 generation and longevity of F_1 generation of *Aphidius colemani (Ac)* when *A. gossypii* was first exposed to *Ac* and then treated with the fungus at 24, 48 and 72 h post exposure in the laboratory.

Treatment	Percent mean (95% CI) parasitism	Mean (95% CI) time from oviposition until parasitoid emergence (days)	Percent mean (95% CI) emergence	Percent mean (95% CI) females F_1 generation	Mean (95% CI) longevity of F_1 generation (days	
					Female	Male
Ac only	56.0 (43.5-67.7)ab	11.0 (10.2-11.8)a	72.1 (58.2-82.9)ab	44.1 (29.6-59.6)a	4.4 (3.8-5.0)a	4.2 (3.5-4.9)a
Ac + LC_{25} (24h)	43.0 (31.5-55.5)b	11.5 (10.7-12.3)a	84.3 (71.3-92.1)a	43.6 (29.2-59.1)a	4.0 (3.4-4.6)a	3.6 (2.9-4.3)a
Ac + LC_{25}(48h)	53.0 (40.7-64.9)ab	11.4 (10.6-12.2)a	90.6 (78.6-96.2)a	43.5 (29.1-59.0)a	4.4 (3.8-5.0)a	4.2 (3.5-4.9)a
Ac + LC_{25} (72h)	71.0 (58.6-80.9)a	12.6 (11.7-13.4)a	71.1 (56.9-82.1)ab	51.1 (36.0-66.1)a	3.7 (3.1-4.3)a	3.7 (3.0-4.4)a
Ac + LC_{50} (24h)	32.0 (21.6-44.5)b	11.4 (10.6-12.2)a	54.2 (40.4-67.7)b	50.0 (30.9-69.1)a	5.0 (3.1-6.9)a	4.2 (3.3-5.1)a
Ac + LC_{50} (48h)	40.0 (28.6-52.6)b	12.2 (11.4-13.0)a	81.2 (67.8-89.9)a	25.4 (14.3-41.0)a	3.7 (3.1-4.3)a	3.9 (3.2-4.6)a
Ac + LC_{50} (72h)	74.0 (61.8-83.4)a	11.0 (10.2-11.8)a	75.5 (61.5-85.6)ab	39.4 (25.6-55.2)a	3.7 (3.1-4.3)a	3.8 (3.1-4.5)a
Ac + LC_{75} (24h)	35.0 (24.2-47.6)b	12.6 (11.8-13.4)a	52.9 (39.0-66.4)b	41.3(27.2-57.0)a	3.8 (3.2-4.4)a	3.5 (2.8-4.2)a
Ac + LC_{75} (48h)	34.0 (23.4-46.5)b	11.7 (10.9-12.5)a	73.9(59.4-84.0)ab	30.0(16.7-47.8)a	3.9 (3.3-4.5)a	4.3 (3.6-5.0)a
Ac + LC_{75} (72h)	54.0 (41.6-65.9)ab	11.5 (10.6-12.4)a	72.9 (58.7-83.4)ab	53.8(38.4-68.5)a	4.3 (3.6-4.9)a	4.0 (3.1-4.9)a

Means within a column followed by different letter differs significantly using Student-Newman-Keuls test ($P < 0.05$).

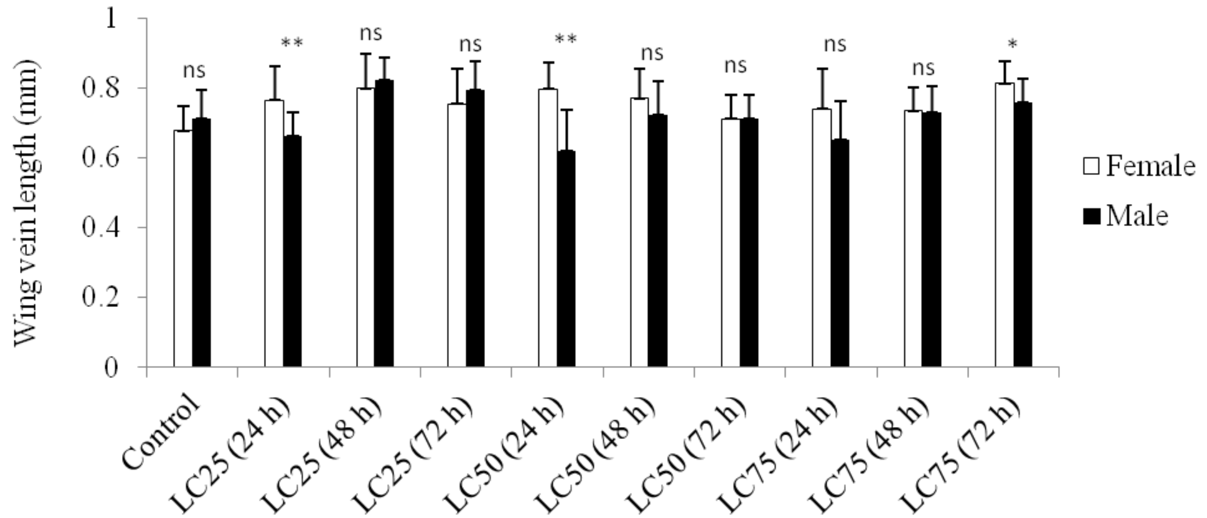

Figure 20. Length of costal wing vein of male and female *Aphidius colemani* that emerged from untreated *Aphis gossypii* and those that had been parasitized and then treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of *Aphis gossypii* (i.e LC_{25}, LC_{50} and LC_{75}) after 24, 48 or 72 h.
*(0.05 > P > 0.01); **(0.01 > P > 0.001); ns = not significant.

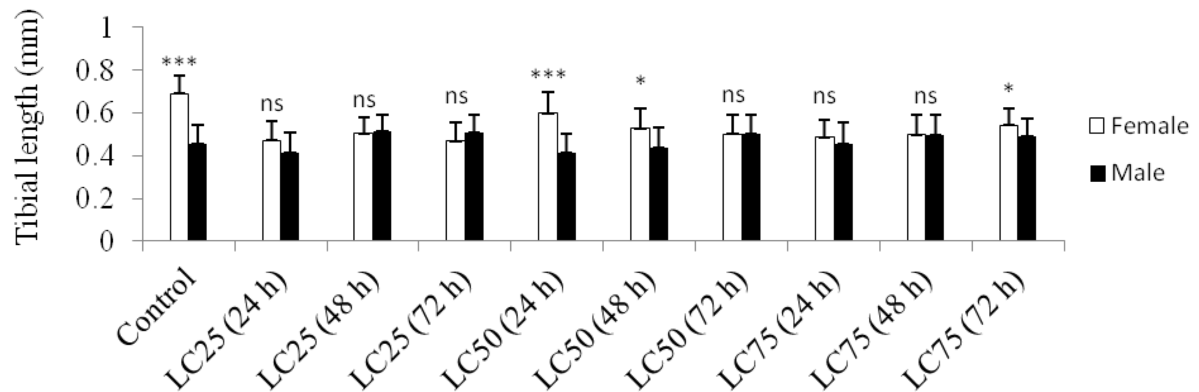

Figure 21. Mean (± SE) length of metathoracic tibia of male and female *Aphidius colemani* that emerged from parasitized and non fungus treated *Aphis gossypii* (control) and those that had been first parasitized and then treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of *Aphis gossypii* (i.e LC_{25}, LC_{50} and LC_{75}) after 24, 48 or 72 h. *(0.05 > P > 0.01); ***(P <0.001); ns = not significant.

7.3.2. *Aphis gossypii* first treated with *M. anisopliae* and then exposed to *A. colemani*

The mean percent of parasitized aphid treated with fungus significantly varied among treatments (F = 5.9; df= 9,40; $P < 0.0001$). However, no significant variation was observed in percent parasitism among aphids treated with different conidial concentration across the exposure time ($P = 0.05$). Percent parasitism relatively declined as the concentration increased with increasing exposure time (Table 10). For example, 29.0% (95% CI = 20.0-41.6) and 20% (95% CI = 11.8-32.0) of parasitized *A. gossypii* were observed when first treated with 1×10^5 conidial ml^{-1} (LC_{25}) and then exposed to *A. colemani* after 24 and 72 h, respectively.

Regardless of the conidial concentration and exposure time of the host to the parasitoids, no significant variation in time from oviposition until emergence, percentage emergence, percentage F_1 female generation, longevity of female and male F_1 generation of *A. colemani,* was observed between fungus treated and control groups ($P = 0.05$) (Table 10).

The length of costal wing vein of *A. colemani* that emerged from *A. gossypii* varied from 0.65± 0.07 to 0.71 ± 0.06 mm for females and 0.68 ± 0.08 to 0.71 ± 0.07 mm for males. The variation was not significantly different when aphids were first treated with fungus and then exposed to parasitoids when compared to untreated control after 24, 48 or 72 h ($P > 0.05$) irrespective of their sex. However, significant variation was observed between male and female *A. colemani* that emerged from *A. gossypii* that had been infected with fungus at higher concentration (LC_{75}) and then exposed to parasitoids during 48 h (t = -4.72; $P < 0.001$) and 72 h (t = -2.67, $P = 0.016$) (Fig. 22).

The tibia length was not significantly different between fungus treated and control groups when aphids were first treated with fungus and then exposed to parasitoid after 24, 48 or 72 h. However, variation was observed between males and females when aphids were first treated with low fungal concentration (LC_{25}) and then exposed to parasitoid after 24 h (t = 2.85, $P = 0.011$) (Fig. 23).

Table 10. Effect of *Metarhizium anisopliae* conidia dispensed at varying lethal concentration that kills *A. gossypii* (LC_{25}, LC_{50} and LC_{75}) on parasitism, emergence, developmental time, female F_1 generation and longevity of F_1 generation of *Aphidius colemani (Ac)* when *A. gossypii* was first treated with the fungus and then exposed to *Ac* at 24, 48 and 72 h post exposure in the laboratory.

Treatment	Percent mean (95% CI) parasitism	Mean (95% CI) time from oviposition until parasitoid emergence (days)	Percent mean (95% CI) emergence	Percent mean (95% CI) females F_1 generation	Mean (95% CI) longevity of F_1 generation (days)	
					Female	Male
Ac only	57.0 (44.5-68.8)a	11.6 (10.9-12.3)a	86.8 (61.9-96.4)ab	53.7 (29.6-76.2)a	3.5 (2.7-4.3)a	4.9 (4.2-5.7)a
LC_{25} + *Ac* (24h)	29.0 (20.0-41.6)b	10.8 (10.1-11.5)a	55.8 (32.7-76.6)ab	41.7 (19.9-67.2)a	4.3 (2.8-5.9)a	4.1 (3.4-4.9)a
LC_{25}+ *Ac* (48h)	22.0 (13.3-34.1)b	12.2 (11.5-12.9)a	47.4 (25.9-69.9)ab	54.2 (27.5-78.6)a	3.8 (2.4-5.1)a	3.3 (2.1-4.4)a
LC_{25} + *Ac* (72h)	20.0 (11.8-32.0)b	11.2 (10.5-11.9)a	63.8 (39.7-82.5)ab	70.8 (41.1-89.4)a	3.8 (2.9-4.7)a	4.0 (3.3-4.8)a
LC_{50} + *Ac* (24h)	30.0 (19.8-42.6)b	11.9 (11.2-12.6)a	72.2 (47.5-88.3)ab	67.5 (38.3-87.4)a	3.9 (3.1-4.7)a	3.4 (2.7-4.2)a
LC_{50} + *Ac* (48h)	20.0 (11.7-32.0)b	11.8 (10.9-12.6)a	60.5 (36.7-80.1)ab	58.3 (30.8-81.5)a	3.4 (2.6-4.3)a	3.5 (2.3-4.7)a
LC_{50} + *Ac* (72h)	17.0 (9.5-28.6)b	10.8 (10.1-11.5)a	91.5 (66.4-98.3)a	94.0 (65.0-99.2)a	3.4 (2.6-4.2)a	3.4 (2.3-4.5)a
LC_{75} + *Ac* (24h)	26.0 (16.5-38.4)b	11.2 (10.5-11.9)a	50.6 (28.4-72.5)ab	70.0 (28.9-93.1)a	4.3 (3.3-5.2)a	4.0 (3.2-4.8)a
LC_{75} +*Ac* (48h)	8.0 (3.3-18.0)b	12.2 (11.5-12.9)a	60.0 (36.3-79.8)ab	83.3 (46.4-96.7)a	4.0 (2.1-5.9)a	3.0 (1.3-4.7)a
LC_{75} + *Ac* (72h)	13.0 (6.6-24.0)b	11.5 (10.7-12.3)a	38.3 (19.0-62.2)b	87.5 (55.8-97.5)a	3.5 (2.7-4.3)a	3.8 (2.7-4.9)a

Means within a column followed by different letter differs significantly using Student-Newman-Keuls test ($P < 0.05$).

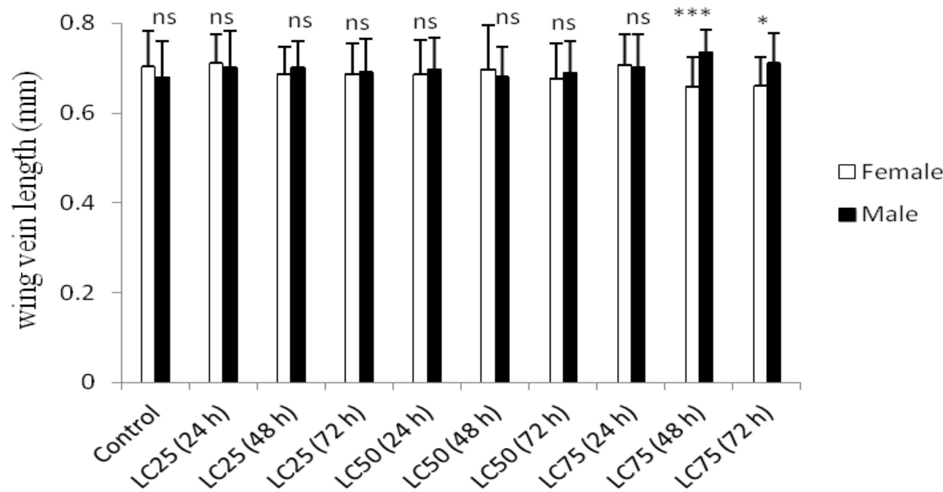

Figure 22. Length of costal wing vein of male and female *Aphidius colemani* that emerged from parasitized and non-fungus treated *Aphis gossypii* and those that had been treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of *Aphis gossypii* (i.e LC_{25}, LC_{50} and LC_{75}) and then parasitized after 24, 48 or 72 h. *(0.05 > *P* > 0.01); ***(*P* <0.001); ns = not significant.

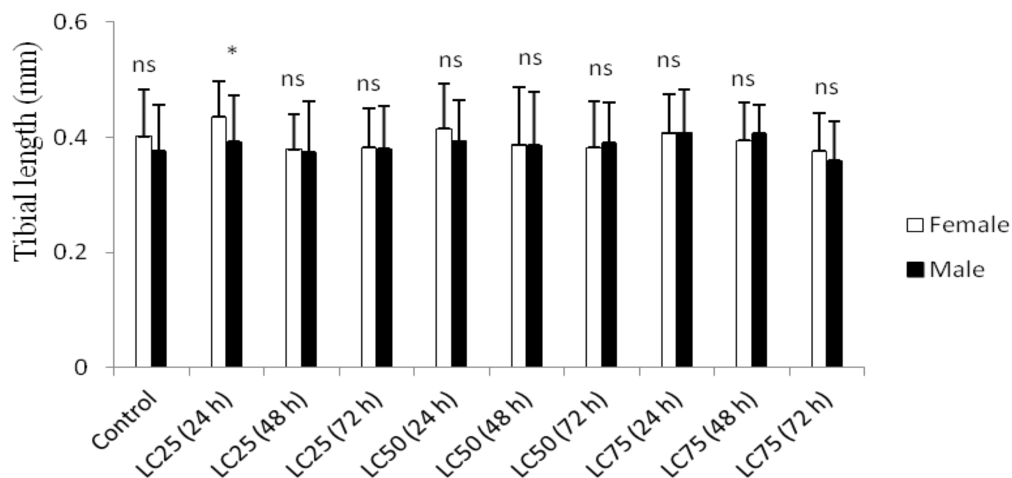

Figure 23. Mean (± SE) length of metathoracic tibia of male and female Aphidius colemani that emerged from parasitized and non-fungus treated Aphis gossypii (control) and those that had been first treated with Metarhizium anisopliae (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of *Aphis gossypii* (i.e LC_{25}, LC_{50} and LC_{75}) and then parasitized after 24, 48 or 72 h. *(0.05 > *P* > 0.01); ns = not significant.

7.3.3. *Brevicoryne brassicae* first exposed to *D. rapae* and then treated with *M. anisopliae*

The percent *B. brassicae* parasitized by female *D. rapae* varied from 12.0 (CI = 5.4-24.5) to 73.0 % (CI = 58.9-83.6) and was significantly different among different treatments, and between treatments and control ($F = 9.4$; df = 9, 40; $P < 0.0001$) (Table 11). However, there was no significant difference in the time from oviposition to emergence between treatments and control, and among different treatments ($F = 1.0$; df = 9, 39; $P = 0.448$) which ranged from 11.2 (95% CI = 10.2-12.2) to 13.0 (95% CI =12.0-14.0) days (Table 11). Moreover, no significant variation between fungus treated and control groups were found in total percentage emergence, percentage of female F_1 generation and longevity of female and male F_1 generation ($P > 0.05$).

The costal wing vein length ranged from 0.69 ± 0.11 to 0.79 ± 0.08 mm for females and 0.62 ± 0.11to 0.82 ± 0.07 mm for males. *D. rapae* emerged from untreated *B. brassicae* and those that had been parasitized and then treated with different concentrations of conidia of *M. anisopliae* after 24, 48 and 72 h; and non-significant difference was observed between the control and treated groups regardless of their sex ($P > 0.05$). However, significant difference was observed in the costal vein length between male and female *D. rapae* from fungus treated at LC_{25} ($t = 3.05$; $P < 0.009$), LC_{50} ($t = 3.69$; $P = 0.003$) after 24 h (Fig. 24).

Tibial length ranged from 0.47± 0.07 to 0.61 ± 0.09 mm for females and 0.41 ± 0.09 to 0.54 ± 0.09 mm for males *D. rapae* that emerged from untreated *B. brassicae* and those that had been parasitized and then treated with different concentrations of conidia of *M. anisopliae* after 24, 48 and 72 h; significant variation was observed between the control and fungus treated groups regardless of their sex ($P < 0.05$). Significant variation in tibial length was also observed between male and female *D.rapae* that emerged from *B. brassicae* that had been parasitized and then treated with fungus at (LC_{50}) ($t = 5.32$; $P < 0.001$), LC_{50} ($t = 2.83$; $P = 0.011$) and LC_{75} ($t = 2.75$; $P = 0.013$) after 24, 48 and 72 h, respectively. However, no variation in tibial length was observed between male and female that emerged from untreated control or the rest of treatment combinations (Fig. 25).

Table 11. Effect of *Metarhizium anisopliae* conidia dispensed at varying lethal concentration that kills *B. brassicae* (LC_{25}, LC_{50} and LC_{75}) on parasitism, emergence, developmental time, female F_1 generation and longevity of F_1 generation of *Diaeretiella rapae (Dr)* when *B. brassicae* was first exposed to *Dr* and then treated with the fungus at 24, 48 and 72 h post exposure in the laboratory.

Treatment	Percent mean (95% CI) parasitism	Mean (95% CI) time from oviposition until parasitoid emergence (days)	Percent mean (95% CI) emergence	Percent mean (95% CI) females F_1 generation	Mean (95% CI) longevity of F_1 generation (days)	
					Female	Male
Dr only	73.0 (58.9-83.6)a	12.4 (11.4-13.4)a	68.7 (50.6-82.5)ab	60.8 (35.2-81.6)a	4.8 (4.2-5.4)a	3.7 (3.1-4.2)ab
Dr + LC_{25} (24h)	27.0 (16.4-41.1)b	11.2 (10.2-12.2)a	53.3 (36.0-70.0)ab	58.3 (30.5-81.7)a	4.7(4.1-5.3)a	4.7(4.2-5.2)a
Dr + LC_{25}(48h)	56.0 (41.9-69.2)ab	12.2 (11.2-13.2)a	76.4 (58.5-88.2)ab	52.8(28.6-75.7)a	4.5 (3.9-5.1)a	4.4 (3.9-4.9)ab
Dr + LC_{25} (72h)	59.0 (44.8-71.9)a	12.0 (11.0-12.9)a	71.0 (52.8-84.2)ab	47.0 (24.1-71.3)a	4.3 (3.8-4.9)a	4.0 (3.5-4.5)ab
Dr + LC_{50} (24h)	23.0 (13.3-36.9)b	11.8 (10.8-12.8)a	43.0 (27.0-60.7)b	83.3 (45.9-96.7)a	4.6 (4.0-5.3)a	4.6 (4.0-5.1)ab
Dr + LC_{50} (48h)	26.0 (15.6-40.1)b	12.0 (11.0-13.0)a	83.1 (66.0-92.7)a	55.8 (31.0-.78.0)a	4.3 (3.8-4.9)a	4.0 (3.5-4.5)ab
Dr + LC_{50} (72h)	59.0 (44.8-71.9)a	12.3 (11.3-13.3)a	77.7 (59.8-89.1)ab	56.3 (31.5-78.4)a	3.9 (3.3-4.5)a	3.6 (3.1-4.1)b
Dr + LC_{75} (24h)	12.0(5.4-24.5)b	11.6 (11.0-13.0)a	53.3 (36.0-69.9)ab	83.3 (45.9-96.7)a	4.3 (3.8-4.9)a	3.8 (3.2-4.3)ab
Dr + LC_{75} (48h)	24.0(14.0-37.9)b	12.0 (10.9-13.1)a	65.9 (47.8-80.3)ab	57.9 (30.1-81.5)a	4.4 (3.8-5.0)a	4.2 (3.7 -4.7)ab
Dr + LC_{75} (72h)	46.0(32.6-60.0)ab	13.0 (12.0-14.0)a	59.9 (42.1-75.5)ab	52.7 (28.6-75.7)a	4.1 (3.5-4.7)a	4.0 (3.4-4.6)ab

Means within a column followed by different letter differ significantly using Student-Newman-Keuls test ($P < 0.05$).

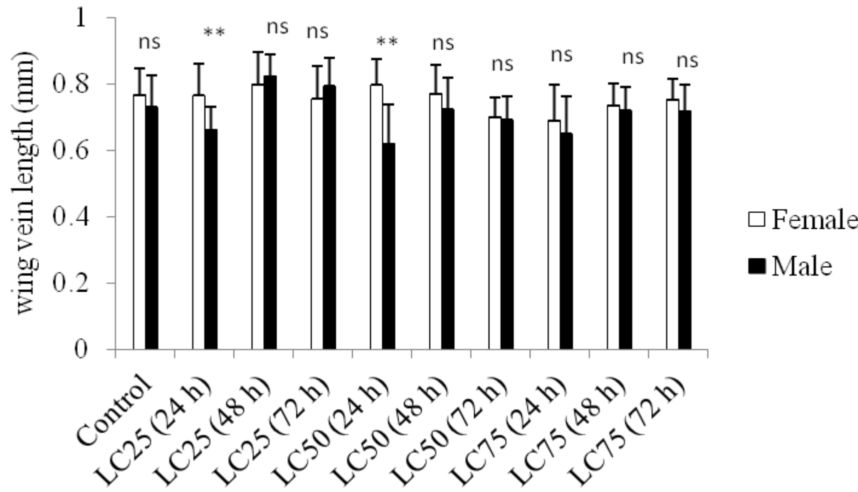

Figure 24. Length of costal wing vein of male and female *Diaeretiella rapae* that emerged from parasitized and non-fungus treated *Brevicoryne brassicae* and those that had been parasitized and then treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concentrat ions that kill 25, 50 or 75% of *Brevicoryne brassicae* (i.e LC_{25}, LC_{50} and LC_{75}) after 24, 48 or 72 h. **(0.01 > P > 0.001); ns = not significant.

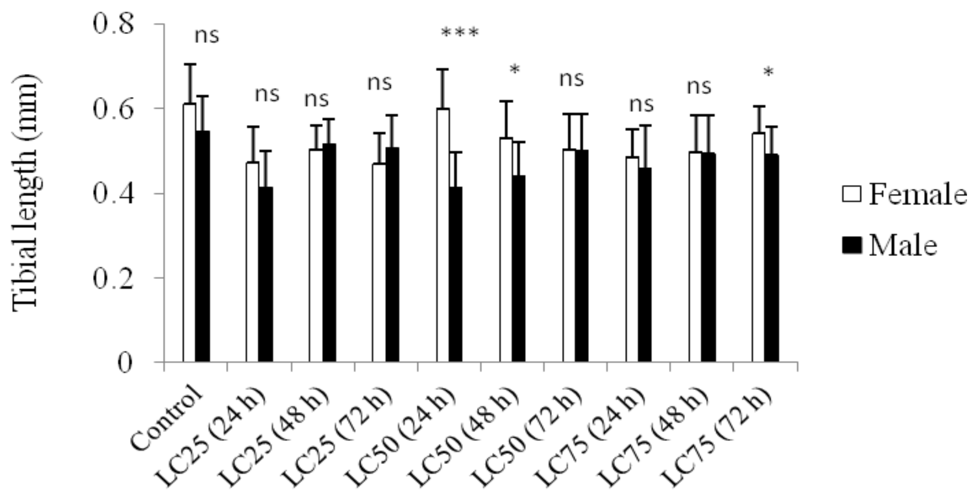

Figure 25. Mean (± SE) length of metathoracic tibia of male and female *Diaeretiella rapae* that emerged from parasitized and non fungus treated *Brevicoryne brassicae* (control) and those that had been first treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of *Brevicoryne brassicae* (i.e LC_{25}, LC_{50} and LC_{75}) and then parasitized after 24, 48 or 72 h. *(0.05 > P > 0.01); ns = not significant.

7.3.4. *Brevicoryne brassicae* first treated with *M. anisopliae* and then exposed to *D. rapae*

The mean percent *B. brassicae* parasitized by female *D. rapae* varied from 12.0% (95% CI = 5.7-23.4) to 76.0% (95% CI = 63.1-85.4) and was significantly different when aphids were first treated with *M. anisopliae* and then exposed to the parasitoid (F = 14.5; df = 9,40; *P* < 0.0001) after 24, 48 and 72 h exposure time (Table 12).

Oviposition to emergence time of F1 generation of *D. rapae* from *B. brassicae* was not significantly different in fungus treated and control groups (F = 1.6; df = 9,38; *P* = 0.16). On the other hand, significant difference in emergence of F1 generation was observed (F = 1.6; df = 9,39; *P* = 0.004), but the variation was not significantly different among fungus treated groups (*P* > 0.05). The proportion of female F1 generation and longevity of female, and male F1 generation did not singnificantly differ between fungus treated and control groups (*P* > 0.05) (Table 12).

The costal wing vein length was ranged from 0.66 ± 0.08 to 0.80 ± 0.09 mm for females and 0.67 ± 0.11 to 0.71 ± 0.06 mm for males *D. rapae* that emerged from untreated *B. brassicae* and those had been treated with different concentrations of conidia of *M. anisopliae* and then parasitized after 24, 48 and 72 h; significant variation was observed between the control and treated groups regardless of their sex (F = 2.67; df = 9,189; *P* < 0.01). Significant difference was also observed in costal vein length between male and female *D. rapae* emerged from aphids treated with fungus and then exposed to parasitoid after 24 h (t =2.65; *P* = 0.019) (Fig. 26).

Tibial length was ranged from 0.41± 0.08 to 0.57 ± 0.14 mm for females and 0.44 ± 0.06 to 0.51 ± 0.08 mm for males *D. rapae* that emerged from untreated *B. brassicae* and those that had been parasitized and then treated with different concentrations of conidia of *M. anisopliae* after 24, 48 and 72 h; variation was observed between the control and treated groups regardless of their sex (F = 4.15; df = 9,189; *P* < 0.01). Significant variation was also observed between male and female *D.rapae* that emerged from *B. brassicae* that had been fungus treated (LC_{75}) and then parasitized after 24 h (t =2.59; df= 16.42; *P* = 0.02) (Fig. 27).

Table 12. Effect of *Metarhizium anisopliae* conidia dispensed at varying lethal concentration that kills *B. brassicae* (LC_{25}, LC_{50} and LC_{75}) on parasitism, emergence, developmental time, female F_1 generation and longevity of F_1 generation of *Diaeretiella rapae (Dr)* when *A. gossypii* was first treated with the fungus and then exposed to *Dr* at 24, 48 and 72 h post exposure in the laboratory.

Treatment	Percent mean (95% CI) parasitism	Mean (95% CI) time from oviposition until parasitoid emergence (days)	Percent mean (95% CI) emergence	Percent mean (95% CI) females F_1 generation	Mean (95% CI) longevity of F_1 generation (days)	
					Female	Male
Dr only	76.0 (63.1-85.4)a	12.2 (11.3-13.1)a	89.4 (71.2-96.6)a	60.0 (38.1-78.6)a	5.1 (4.5-5.7)a	4.1 (3.5-4.7)a
LC_{25} + *Dr* (24h)	35.0 (23.7-48.3)bc	11.6 (10.7-12.5)a	51.4 (33.2-69.2)b	45.0 (20.9-71.8)a	4.1 (3.4-4.9)a	4.0 (3.3-4.7)a
LC_{25}+ *Dr* (48h)	18.0 (10.0-30.3)c	11.2 (10.3-12.1)a	51.7 (33.4-69.5)b	58.3 (30.9-81.5)a	3.9 (3.2-4.5)a	3.9 (3.2-4.6)a
LC_{25} + *Dr* (72h)	65.0 (51.7-76.3)ab	11.3 (10.4-12.2)a	64.6 (45.4-80.1)ab	52.5 (31.6-72.6)a	4.0 (3.4-4.6)a	3.8 (3.1-4.4)a
LC_{50} + *Dr* (24h)	19.0 (10.7-31.4)c	11.9 (11.0-12.8)a	72.7 (53.3-86.1)ab	38.9 (16.7-66.9)a	3.6 (3.0-4.3)a	4.1 (3.5-4.7)a
LC_{50} + *Dr* (48h)	12.0 (5.7-23.4)c	11.2 (10.3-12.1)a	56.7 (37.9-73.7)ab	66.7 (37.7-86.9)a	4.6 (4.0-5.3)a	4.4 (3.7-5.2)a
LC_{50} + *D r*(72h)	50.0 (37.2-62.8)b	12.8 (11.9-13.7)a	70.3 (50.9-84.4)ab	64.8 (42.5-82.1)a	4.2 (3.6-4.9)a	4.3 (3.6-5.0)a
LC_{75} + *Dr* (24h)	14.0(7.1-25.8)c	11.5 (11.5-13.5)a	86.6 (68.2-95.2)ab	52.5 (20.9-74.6)a	4.2 (3.6-4.9)a	3.9 (3.1-4.6)a
LC_{75} + *Dr* (48h)	13.0(6.4-24.6)c	11.0 (10.0-12.0)a	81.3 (59.5-92.7)ab	38.9 (16.7-66.9)a	4.5 (3.8-5.2)a	3.9 (3.3-4.5)a
LC_{75} + *Dr* (72h)	26.0(16.2-39.0)c	11.2 (11.1-12.9)a	43.3 (26.3-62.1)b	58.3 (34.2-79.0)a	4.3 (3.6-4.9)a	4.1 (3.6-4.8)a

Means within a column followed by different letters differ significantly using Student-Newman-Keuls test ($P < 0.05$).

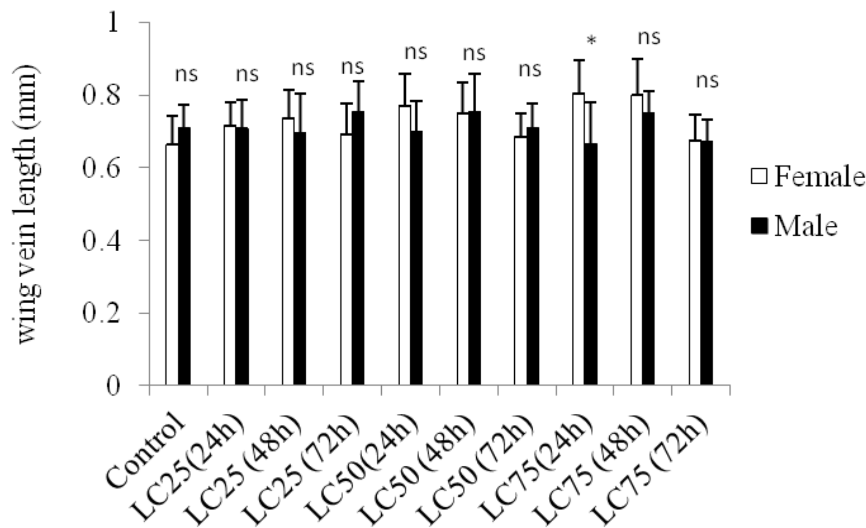

Figure 26. Length of costal wing vein of male and female D. rapae that emerged from parasitized and non-fungus treated B. brassicae and those that had been treated with M. anisopliae (ICIPE 62) at lethal concentrations that kill 25, 50 or 75% of B. brassicae (i.e. LC_{25}, LC_{50} and LC_{75}) and then parasitized after 24, 48 or 72 h. *($0.05 > P > 0.01$); ns = not significant.

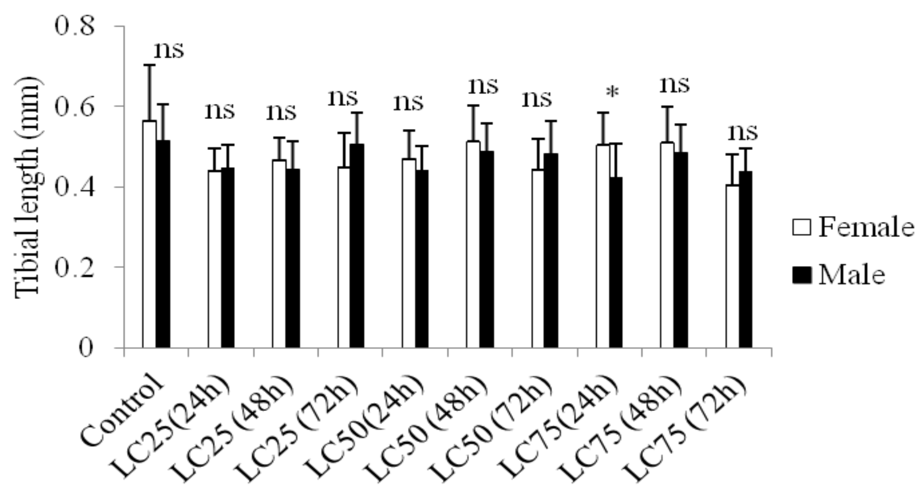

Figure 27. Mean (± SE) length of metathoracic tibia of male and female *Diaeretiella rapae* emerged from parasitized and non-fungus treated *Brevicoryne brassicae* (control) and those that had been first treated with *Metarhizium anisopliae* (ICIPE 62) at lethal concen trations that kill 25, 50 or 75% of *Brevicoryne brassicae* (i.e. LC_{25}, LC_{50} and LC_{75}) and then parasitized after 24, 48 or 72 h. *($0.05 > P > 0.01$); ns = not significant.

7.4. Discussion

Metarhizium anisopliae ICIPE 62 is a candidate isolate for the management of *A. gossypii, B. brassicae* and *L. pseudobrassicae* (Chapter 3). This isolate was also found to be less detrimental to adult *C. lunata* and warrants integration into management programs targeted at the three aphid species (Bayissa *et al*., 2016). The results of interaction between *M. anisopliae* ICIPE 62 and parasitoids in the current study also indicated no significant effect of the fungus on most of the parameters measured for F_1 generation of *A. colemani* and *D. rapae* when the host aphids were exposed to varying conidia concentrations of the fungus and subsequently exposed to the parasitoids, and vice versa.

The results showed that parasitism observed between the control and the treatments was not significant when *A. gossypii* was exposed to *A. colemani* then sprayed with fungus regardless of the exposure time and conidial concentration; this was observed in *B. brassicae* exposed to *D. rapae* then sprayed after 24 h with low conidial concentration at 24 and 48 h post exposure and with higher conidial concentrations. This demonstrates the importance of exposure time to the parasitoid and the interaction with the fungus if these two control agents have to be deployed together for the management of the pest. On the other hand, concentration had significant role in the interaction between *D. rapae* and the fungus. Similar findings on the effect of time on parasitism were reported by Mesquita and Lacey (2001) and Martins *et al.* (2014) who studied interactions between *P. fumosoroseus and Aphelinus asychis* Walker on *D. noxia* and, *B. bassiana* and *D. rapae* on *M. persicae,* respectively.

The reduction in the parasitism rate on previously fungus infected *A. gossypii* and *B. brassicae* is probably due to less acceptability and suitability of the infected host by the parasitoids. This finding is in accord with Martins *et al.* (2014) though this was not the case with *B. brassicae* treated with lower conidial concentration that has been parasitized by *D. rapae* after 72 h. Baverstock *et al.* (2005) suggested that parasitoids may detect infective conidia

on their host and could affect the decision of the parasitoid whether to oviposit in an infected host or to avoid it. It is also possible that the antagonistic interaction between the aphid host and the fungus resulted in rapid death of the aphid and reduced the parasitism rate.

The absence of significant difference between the fungus treated and untreated control in emergence and developmental time observed for both *A. colemani* and *D. rapae* was probably due to fungistatic substance secreted by the parasitoid. Willers *et al.* (1982) reported that parasitoids secret fungistatic substances into the hemolymph of the host that impede colonization by the fungus and consequently promotes normal development and emergence of the parasitoid.

The decreased emergence of *D. rapae* when its host aphids are treated with higher conidial concentration (LC_{75}) for 72 h before parasitism suggests the lack of survival of the host to support the parasitoid. Previous observation opined that survival of parasitoid developing in fungus infected host depends on the time interval between parasitoid oviposition and infection by the pathogen (Brooks, 1993).

The sex ratio of F_1 generation of *A. colemani* and *D. rapae* was not altered in response to host aphids infection by *M. anisopliae*. This is a positive result for the potential of compatible utilisation of the EPF and the two parasitoids in the IPM of aphids. On the other hand, the differences observed in tibial length and costal vein length between male and female could be more of biological effect than treatment related effect.

6.5. Conclusions

In conclusion, the inoculation of *A. gossypii* and *B. brassicae* with sublethal and lethal concentration of *M. anisopliae* isolate ICIPE 62 have no significant harmful effect on the development and survival of *A. colemanii* and *D. rapae*.

However, the parasitism rate depends on the time of exposure of the aphids to the fungus and the parasitoid. On the other hand, sex ratio was not affected by the treatment which should be advantageous to the overall aphid suppression strategy. In general, and based on the different parameters measured in this study the use of *M. anisopliae* isolate ICIPE 62 together with the two important parasitoid species justifies their co-utilization within the context of IPM. However, additional studies encompassing screenhouse and field studies are warranted to generate more information on the interaction among fungal pathogen, parasitoids and aphid pests before recommendation to vegetable growers.

CHAPTER EIGHT: EFFECTIVENESS OF *METARHIZIUM ANISOPLIAE* IN THE CONTROL OF APHIDS ON CRUCIFER AND OKRA CROPS UNDER SCREENHOUSE AND FIELD CONDITIONS

8.1. Introduction

In many African countries, the horticultural sub-sector contributes significantly to development of the economy (Nyambo and Verschoor, 2005; Ekesi *et al*., 2009; HCDA, 2009; Ekesi, 2010). Despite the importance, production has been hampered largely by biotic constraints attributed to arthropod pests. For example, on vegetables, aphids are widely recognized as economically damaging pests of crucifers (Blackman and Eastop, 2002; Van Emden and Harrington, 2007). In Kenya, cabbage aphid (*B. brassicae*) and turnip aphid (*L. pseudobrassicae*) are among the economically important species on kale and cabbage (Oruku and Ndun'gu, 2001). The melon/cotton aphid (*A. gossypii*) is also widely recognized as a key pest of okra in most of sub-Saharan Africa (Seif, 2002; Nderitu *et al*., 2008; Saethre *et al.,* 2011; Amoakwah *et al.,* 2013). Their effect on productivity and quality of plants are through direct feeding damage, contamination with sugary secretions, and vectoring of plant pathogens. Their high fecundity and short generation time can also exacerbate damage on crops.

Vegetable producers in the region rely heavily on the use of synthetic chemical pesticides with little guidance on handling and use (Ntow *et al*., 2006; Grzywacz *et al*., 2010). This in turn results in human and environmental contamination, toxic residues on the produce and disruption of useful natural enemies. Additionally, insecticide resistance has been reported for several species of aphids including *A. gossypii* (Foster *et al*., 2007. Carletto *et al*., 2010; Koo *et al*., 2014).

Entomopathogenic fungi mainly belonging to two phyla, the Zygomycota and the Ascomycota are known to attack several arthropod pests including aphids. At least 170 fungal-based bio-insecticides and bio-acaricides have been

developed worldwide (Faria and Wraight, 2007). South and Central America has 50% share of this figure, with Africa accounting for only 3% of the biopesticide products developed globally (Faria and Wraight, 2007). Several commercial products based on *M. anisopliae, B. bassiana*, and *Isaria* species have been registered for aphid control in Europe and North America, (Jandricic *et al*, 2014). However, similar products are lacking in Africa. In previous studies, we have identified *M. anisopliae* isolate ICIPE 62 as a candidate biopesticide for the management of three aphid species, namely *B. brassicae, L. pseudobrassicae* and *A. gossypii* (see Chapter 4 and 5). Additionally, results from interaction studies showed that the isolate has no adverse effect on naturally occurring aphid parasitoids (*D. rapae*, *A. colemani*) and predator (*C. lunata*) (Bayissa *et al*., 2016). In this study, (1) the efficacy of two formulations of *M. anisopliae* ICIPE 62 in suppressing the three aphid species (*A. gossypii* and *B. brassicae, L. pseudobrassicae*) on okra and crucifer was evaluated under screenhouse and open field conditions; and (2) the impact of the isolate on aphid natural enemies was also assessed.

8. 2. Materials and Methods

8.2.1. Description of study site

The screenhouse study was carried out at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville Campus, Nairobi, Kenya (03.35517° S and 037.33861° E) and the field trials were undertaken at the *icipe*, Thomas Odhiambo Campus (ITOC), Mbita Point field station, Western Kenya (0°34′ S and 34°10′ E;1170 m.a.s.l.). The area is characterized by tropical climatic condition with an average minimum and maximum temperatures of 16 and 28°C, respectively, based on data obtained at ITOC in 2014). The area also recieves bimodal rains (March-July and September-December) with an average annual raifall of 1150 mm.

8.2.2. Production of fungus inoculum for screenhouse and open field trials

Conidia were produced in liquid and solid phase. The medium for liquid phase consisted of 5 g Yeast Extract, 15 g Glucose, 5 g Peptone and 500 ml sterile distilled water autoclaved for 20 min at 121°C. Conidia were harvested from 2-3 weeks old sporulating cultures and suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads (3 mm). The suspension was vortexed for 5 minutes at 700 rpm and quantified using haemocytometer under light microscope. After the medium had cooled to 28°C, 5 ml of conidia were titrated to 1×10^8 conidia ml $^{-1}$ and later transferred to 250 ml flask containing 50 ml of sterile liquid medium. The culture was incubated in a rotary shaker at 100 rpm at 25^0C for 5 days to obtain blastospores. Dry conidia were produced on long rice substrate as previously described (Maniania, 1993a and Ekesi *et al.*, 2001). Two kilogram of long-grain rice substrate was transferred in polyethylene bag and then autoclaved for 60 min at 121°C. The substrate was then allowed to cool for up to 28°C and inoculated with the 50 ml of 5 day-old culture suspension of blastospore. The bag was sealed under aseptic condition and incubated for three weeks at ambient temperature of 20 - 26°C and 40 - 70% RH (Maniania, 1993a and Ekesi *et al.*, 2001). After three weeks, the polyethylene bag was removed and the content transferred to sterile plastic buckets (33 x 25 x 13 cm) to allow the culture to dry for 5 days at room temperature before harvesting. Conidia were harvested by sifting using 295 µm mesh size and stored at 4°C for use in the screenhouse or field experiment. Viability was assessed by spread-plating 0.1 ml of conidia titrated to 1×10^6 conidia ml^{-1} on SDA plates before application. Conidia with viability above 90% were used for the various experiments.

8.2.3. Screenhouse experiment

Kale and okra were planted in plastic pots (15 × 20 cm) by direct seeding. Plants were thinned to one plant per pot when they were ca. 20 cm tall and transferred to sleeved cages (100 × 100 cm). Each caged plant was then

artificially infested with 100 apterous adult aphids of uniform age and thereafter transferred into a screenhouse (5 × 10 m). The prevailing temperature and relative humidity (rh) in the screenhouse during the experimental period ranged from 15-29°C and 30-90% RH, respectively, with photoperiods of 12: 12 L:D. The insects were allowed to multiply for 8 days to ensure that they reached a stable age distribution (Banken, 1996; S. Ekesi, unpublished) which was the only basis on which different values for rates of increase could be compared (Birch, 1948). The initial aphid density (N_o) before fungus application was determined by destructive sampling of 5 plants for each aphid species.

For each aphid species, treatments were arranged in complete randomized design (CRD) and replicated four times. Conidial suspension titrated to 1.0 × 10^8 conidia ml^{-1} was formulated in water (Triton X-100+water at the ratio of 0.05:99.95) and emulsifiable formulation (Triton X-100 + Corn Oil + Water at the ratio of 0.05: 0.1:99.85). Nutrient agar (0.1%), glycerin (0.1%) and molasses (0.5%) were added to each formulation as protectants to complete the formulations (Maniania, 1993b). Each infested plant was then sprayed with 20 ml of each formulation using a hand sprayer fitted with a hollow cone nozzle with a volume diameter droplet of 41 µm. Control groups were treated with either sterile distilled water containing 0.05% Triton X-100 or 0.1% oil plus the protectant ingredients listed above.

The efficacy of fungus application on aphids was based on mortality caused by the fungus on randomly selected aphid samples and aphid population growth rate (Birch, 1948). Twenty (20) apterous adult aphids were gently picked at random using a camel hair brush from treated plants and placed individually in screened transparent plastic vials (50 x 100 mm) and fed with surface-sterilized kale or okra leaf disks. Insects were maintained in a controlled environment room (26 ± 2°C, 60 ± 5% RH, and photoperiod of 12:12 L: D). Mortality was recorded daily for 7 days and dead insects were processed as described in section 3.2.3. To determine aphid population growth rate on the potted plants

following spray application of the fungus, all potted plants were removed from the cages after 7 days and all the aphids on the plants counted. The time interval of 7 days was chosen because it provided enough time for population growth to occur, but not enough time for the aphids to kill the plants.

8.2.4. Field experiments

Field experiments were conducted at (ITOC), Mbita Point field research station, Western region of Kenya during two consecutive seasons (March – June 2014 and July – October 2014).

8.2.4.1. Nursery, seedling establishment and experimental design

Kale, 'var. 1000-headed' was seeded on a raised seedbed and then transplanted to the experimental plots (10 × 6 m) after four weeks with an inter-row and intra-row spacing of 60 cm and 45 cm, respectively. A 2 m gap between each plot and 3 m between each block were maintaind to separate each treatment (Figs 32 and 33). Calcium ammonium nitrate (CAN) fertilizer was applied at the rate 5 g per plant at 3 weeks after transplanting. Okra 'var. Pusa sawani' was directly seeded in rows on the plots (10 × 6 m) at 40 cm inter-row and 30 cm intra-row spacing. A 2 m gap was also maintained between plots and 3 m between blocks to separate the treatments. Diammonium phosphate (DAP) was applied to the plots at the recommended rate of 125 kg ha^{-1} and mixed well with soil before sowing the okra seeds.

Plots were weeded manually and irrigated when necessary using overrheaded sprinkler irrigation. The treatments were arranged in a randomized complete block design with four replications. For spray application, conidia of *M. anisopliae* were suspended in water containing 0.1% corn oil, nutrient agar (0.1%), glycerin (0.1%), Tween-80 (0.05%) and molasses (0.5%) as protectants to complete the formulations (Maniania, 1993b). The fungus was applied at 1×10^{12} conidia ha^{-1} and 5×10^{12} conidia ha^{-1} in both kale and okra plots. Control

plots were sprayed with water containing 0.05% Tween-80, 0.1% corn oil plus the protectant ingredients as described in section 7.2.3. Lambda (L)-cyhalothrin (Karate®) was also applied as standard check at the recommended rate of 17.5 g a.i ha^{-1}. Treatment application was performed starting from 35 days after transplanting (DAT) of the seedlings of kale plants and 40 days after emergence (DAE) of okra plants, and repeated five times at 5 days interval. Treatments were applied late in the evening (1700-1800 h) to lessen the adverse effect of ultraviolet radiation (Moore and Prior, 1993). Both biological and chemical insecticides were applied using CP 15® knapsack (Cooper Pegler, Sussex, UK) at sprayer output of 400 litres ha^{-1}.

8.2.4.2. Aphids infestation and yield assessment

Key insect pests were aphids and flea beetles (Figs. 28 and 29). Data on the number of the key pests and their damage level on the plants were collected starting from 35 DAT of kales and 40 DAE of okra plants as described below:

Aphids: Aphid were counted on 3 leaves per plant (1 from lower, 1 from top and 1 from apex) of 20 randomly selected plants from each treatment plot. Aphid counts were done *in situ* at 5 days interval starting from 35 DAT and 40 DAE for kale and okra plants, respectively. Mortality of aphids due to treatment effect was determined by gently picking 30 aphids using a camel hair brush randomly from fungus and control treated plots at 12 h post treatment. The aphids were then placed individually in screened transparent plastic vials (50 x 100 mm) and fed with surface-sterilized kale or okra leaf disks. Mortality was recorded daily for 5 days and dead insects were processed and maintained as described in section 4.2.3.

Predators: The number of both adults and larvae of Coccinellidae (ladybird beetles), Syrphidae (hover flies), Chrysopidae (lacewings) and other arthropod predators were counted on two central rows in each treatment plot on the same

dates when aphid density was assessed. Counting was done early in the morning between 6:30 and 8:00 am when they were less active.

Parasitoids: The number of parasitized aphids were estimated by counting mummies on the randomly sampled 20 plants in each plot on the same dates when aphids density was assessed.

Other insect pests: Although the target pest in this study was aphid species, field observations have shown rising importance of the flea beetles (*Podagrica* spp.) in okra plots. The number of insects present on the 20 randomly selected okra plants was counted from each treatment plot. Counting was done early in the morning between 6:30 and 8:00 am when the flea beetles were less active on the same dates when predator density was assessed over the sampling periods.

Yield assessment: Yield data was collected from 10 plants randomly selected from the 2 middle rows of each treatment plot (both okra and kale). Okra pods were picked from the selected plants when they had reached commercial maturity stage, and kale leaves were picked when fully opened from each plant at 4 days interval. Both the leaves and pods were further sorted into marketable (aphid free and without blemishes) and non-marketable (with aphids and blemishes) and weighed. The weight was extrapolated into kilogram per hectare (Kg ha^{-1}).

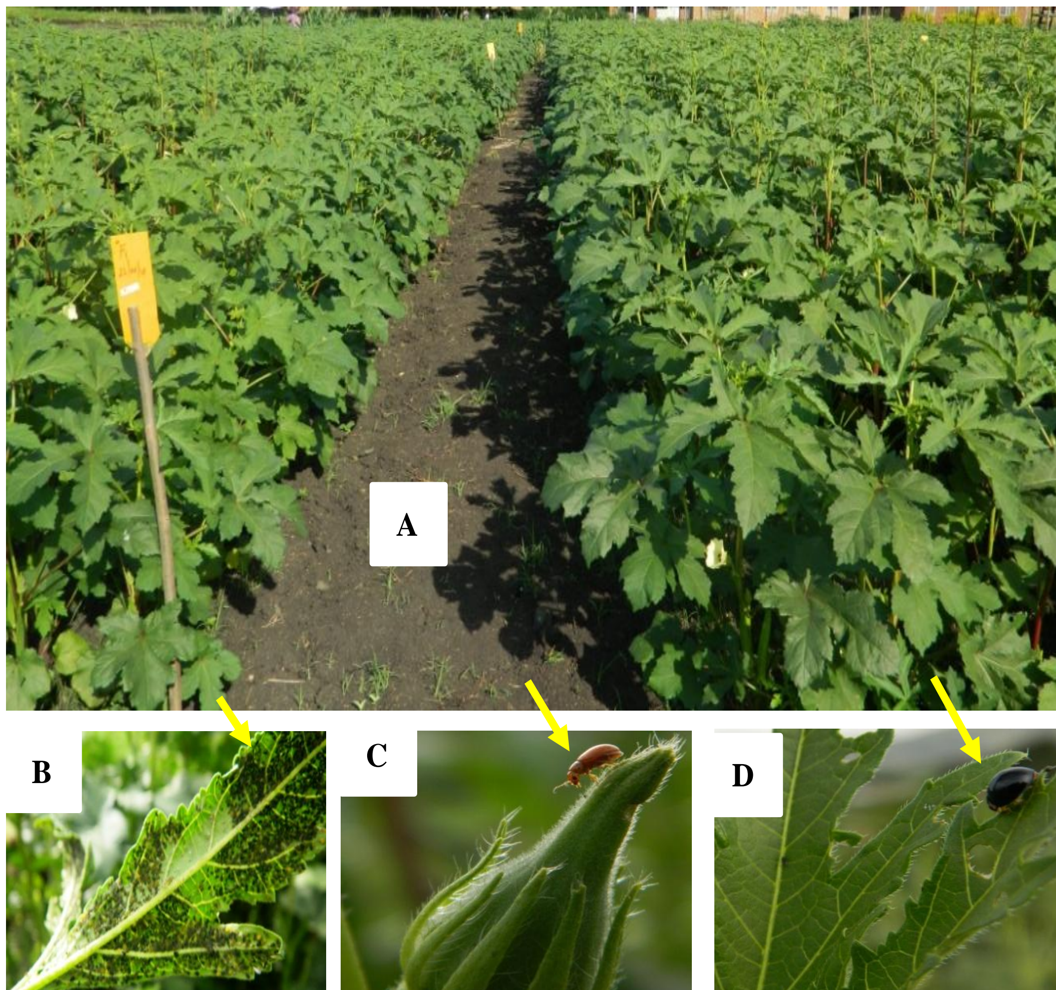

Figure 28. Okra field trial showing treatments arrangement across experimental plots (A); *Aphis gossypii* devastating okra leaf (B); Flea beetle attacking pod (C); Coccinellid hunting for aphids (D). Photos: W.H. Bayissa.

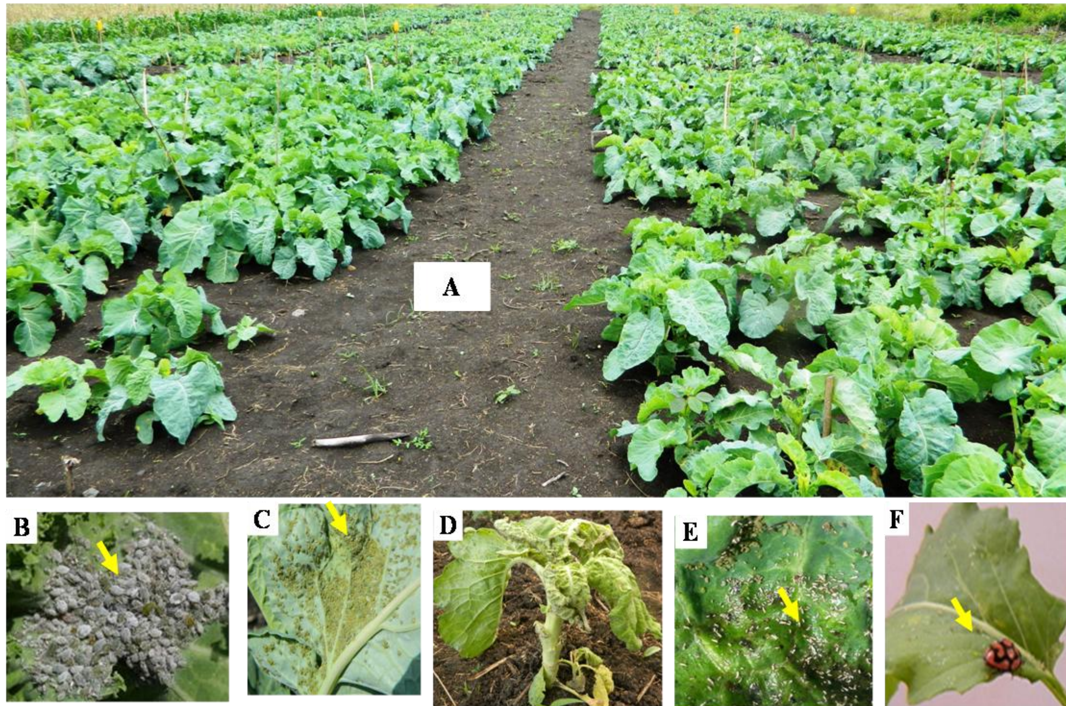

Figure 29. Kale field trial showing treatments arrangement across experimental plots (A); *Brevicoryne brassicae* devastating kale leaf (B); *Lipaphis pseudobrassicae* attacking kale leaf (C); heavily infested plant dying (D); leaf contaminated with honeydew produced by aphids (E); Coccinellid hunting for aphids (F). Photos: W.H. Bayissa.

8.2.4.3. Statistical analysis

All data analysis was performed using R v2.14.2 statistical software package (R Development Core Team, 2011). Screenhouse data on aphid mortality was analyzed using a generalised linear model (GLM) with binomial regression analysis. Data on the number of aphids/plant was fitted to GLM using negative binomial regression analysis. The final and initial aphid numbers/plant were compared using multiple comparisons based on the model parameter estimates.

The aphid population growth rate was also determined as the instantaneous rate of increase (r_i) for each treatment using the equation:

$$ri = \frac{\ln\left(\frac{Nt}{No}\right)}{t}$$

where N_o is the initial number of aphids in the population, and N_t is the number of aphids in the population at the end of the time interval, t (in days) (Walthall and Stark, 1997; Stark and Banks, 2003). "Positive values of r_i indicate a growing population; when r_i is zero, the population is neither growing nor declining, and when r_i is negative, the population is on the decline and headed towards extinction" (Stark and Banks, 2003). The data on r_i were subjected to ANOVA and means were compared using Tukeys HSD test.

Field data on aphids and flea beetles count were analyzed separately by generalized linear mixed models (GLMM) using a function 'lmer' from lme4 package and other packages including MASS (R Development Core Team, 2011). Generalized mixed model was chosen based on its appropriateness to handle variability over a period of time other than the treatments effects without transforming the data. Therefore, plots from which plants were sampled over a period of time in the field system were included in the model as a random and the experimental treatments as fixed effects. Sampling was not made on apex leaves of kale at 35, 40 and 45 DAT of kale; therefore, it was removed from the analysis. Data on percent mortality were fitted to generalized linear model using binomial distribution. Count data on natural enemies and other pests were subjected to generalized linear model using Poisson distribution with a log link function. The yield data for each season was analyzed separately using ANOVA and means were compared using Student-Newman-Keuls test. Count data on non-target organisms was analyzed using GLM with Poisson regression analysis. Post-spray samples of non-target organisms were pooled over the entire season.

8.3. Results

8.3.1. Efficacy of *Metarhizium anisopliae* on aphids in the screenhouse

Infection by *M. anisopliae* was observed in all the adult aphid species treated with conidial suspension in the screenhouse and collected after spray application and processed in the laboratory. In *A. gossypii*, mortality in the control (without conidia) cages was 13.8 and 17.5% in aqueous and emulsifiable formulations, respectively. In fungal treated cages, mortality was 72.7 and 93.8% in conidia formulated in aqueous and emulsifiable formulations, respectively (Fig. 30 A). In *B. brassicae*, mortality in the control treatments was 18.8 and 20.0% in the aqueous and emulsifiable formulations, respectively. In the fungal treatment, mortality was 89.4 and 95.0% in the aqueous and emulsifiable formulation treatments, respectively (Fig. 30 B). For *L. pseudobrassicae,* control mortality was 15.0 and 18.8% in water alone and 0.1% oil alone, respectively; while fungal treated aphids mortality was 90.8 and 85.2% in aqueous and emulsifiable formulations, respectively (Fig. 30 C).

There was a significant interaction between aphid species and formulations ($P < 0.05$). Significant variation was also observed in conidia formulated as aqueous and emulsifiable form with regard to mortality on *A. gossypii* ($P = 0.03$) (Fig. 32 A). However, in the control treatments, there was no significant difference between aqueous and emulsifiable formulations across the three aphid species ($P \leq 0.05$) (Fig. 30 A, B and C). Mortality induced by conidia formulated in emulsifiable and aqueous formulations on either *B. brassicae* or *L. pseudobrassicae* were not significantly different ($P < 0.05$) (Fig. 30).

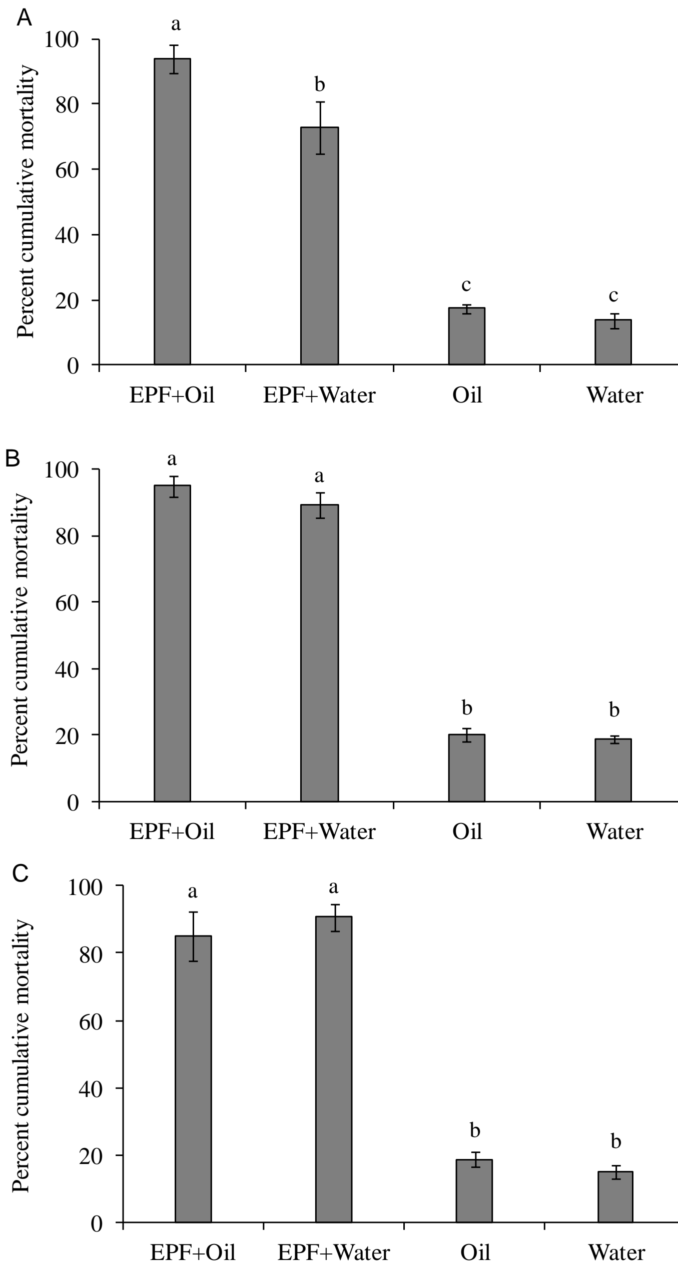

Figure 30. Percent mortality of *A. gossypii* (A), *B. brassicae* (B) and *L. pseudobrassicae* (C) collected from screenhouse and processed in the laboratory 7 days after treatment with conidia of *Metarhizium anisopliae* isolate in oil (EPF + Oil) and aqueous (EPF + Water) formulation, and oil alone and water alone.

The average initial aphid density (N_o) before fungus application was determined by destructive sampling of 5 plants for each species and the results were as follows: 342 for *B.brassicae*, 509 for *L. psudobrassicae* and 251 for *A. gossypii*. The number of aphids per plant declined following application of aqueous and emulsifiable formulations, while it increased in the control treatments for all species (Fig. 31) with visible effect on the plants growth (Annex 1 A and B). There was, however, significant variation (P = 0.002) between the three aphid species with emulsifiable formulation of conidia. **S**ignificant variation was also observed between formulations (χ^2 = 85.04; df = 4; P < 0.0001) and aphid species (χ^2 =149.6; df=2; P < 0.0001).

Comparison between the final and initial aphid numbers/plant also showed significant variation for all the three aphid species treated with conidia formulated in oil (P = 0.002). However, there was no significant difference between initial and final aphid numbers/plant in both oil alone (P = 0.9) and water alone (P = 0.5) as control treatments. On the other hand, variation was observed in the number of aphids/plant in the water control and conidia formulated oil (P < 0.001), water control and conidia formulated as aqueous (P = 0.004), and oil control and conidia formulated in oil (P < 0.001) at 7 days post treatment. However, there was no significant difference between conidia formulated as emulsifiable and aqueous (P = 0.41) (Fig. 31). Variation was also observed in the number of aphids/plant within individual species, *A. gossypii* (χ^2 = 98.06; df = 4; P < 0.0001), *B. brassicae* (χ^2 = 85.04; df = 4; P < 0.0001) and *L. pseudobrassicae* (χ^2 = 22.78; df = 4; P = 0.0001) due to treatment (Fig. 31).

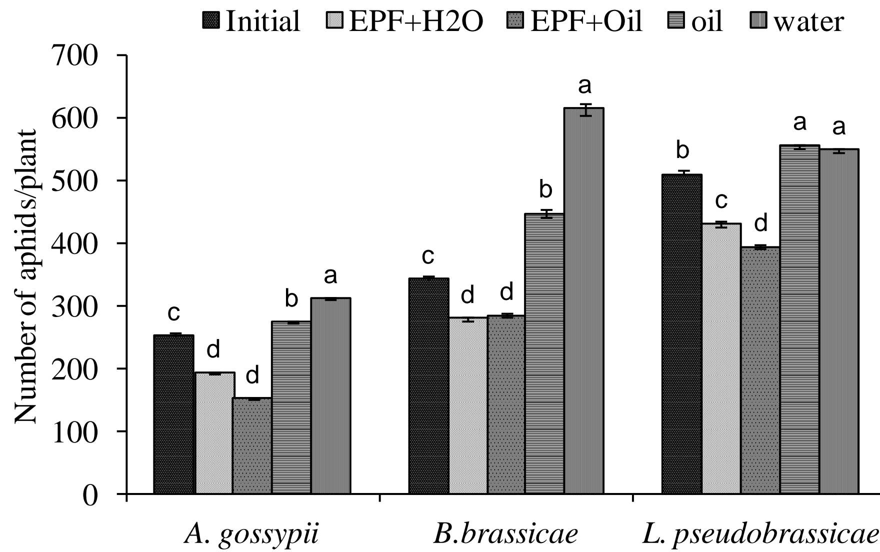

Figure 31. Average number of aphids/plant before treatment (initial) and 7 days after treatment with conidia of *Metarhizium anisopliae* ICIPE 62 formulated in aqueous (EPF + water) and oil (EPF + Oil), oil alone and water alone. Bars indicate means SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments by Tukey's HSD multiple range test at *P*-values of < 0.05.

The r_i values of aphid populations following the application of two formulations of *M. anisopliae isolate* ICIPE 62 are presented in Fig. 38. Conidia formulated in emulsifiable and aqueous formulations resulted in negative r_i values, while the r_i values were positive in the control treatments. Overall, both conidial formulations had significant effect on aphid population growth compared to controls ($P < 0.05$) (Figs. 32 A, B and C).

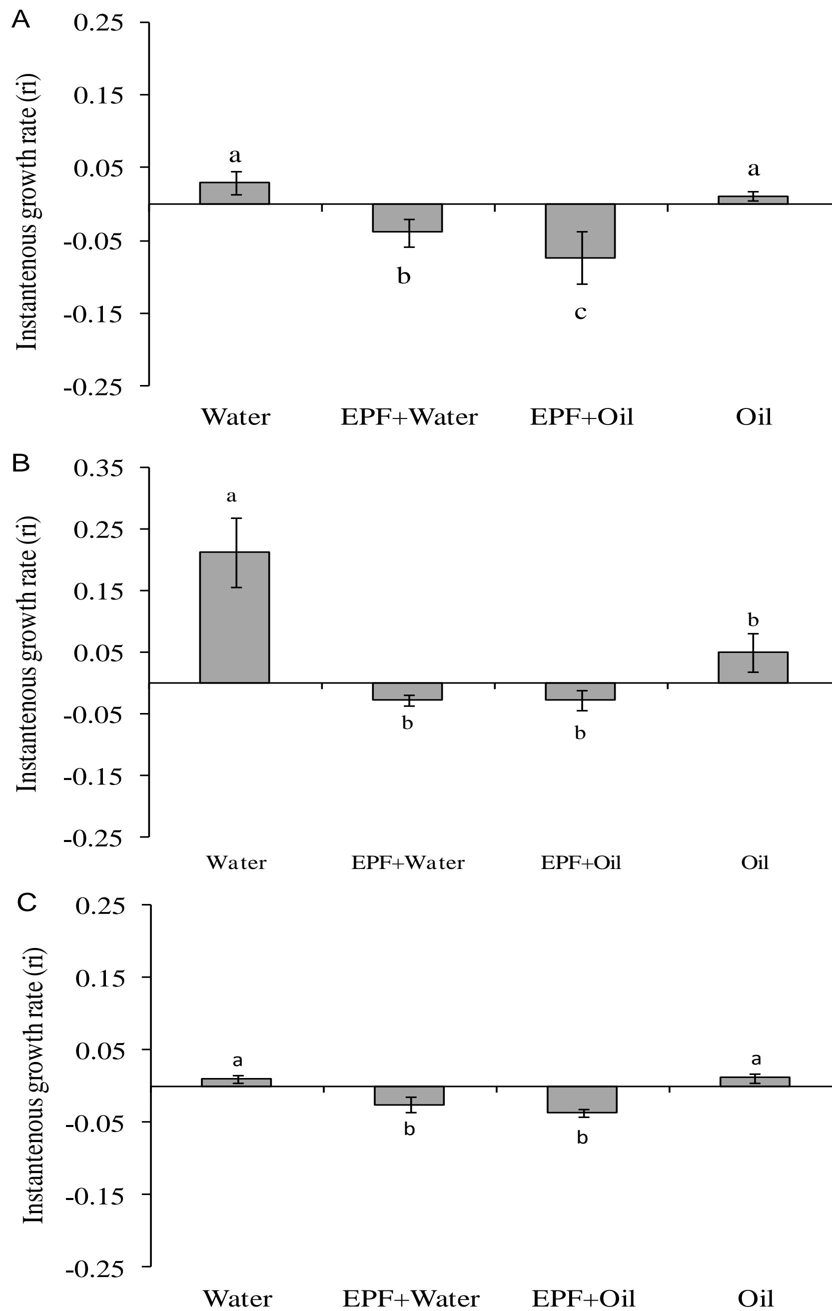

Figure 32. Instantaneous rate of increase of three aphid species. (A) *A. gossypii*, (B) *B. brassicae* and (C) *L. pseudobrassicae* 7 days after treatment with conidia of *Metarhizium anisopliae* ICIPE 62 formulated in aqueous (EPF + Water) and oil (EPF + Oil), oil alone. Bars indicate means SE at 95% CI. Means followed by the same letter indicate no significant differences between treatments by Tukey's HSD multiple range test at *P*-values of < 0.05.

8.2.2. Field efficacy of *Metarhizium anisopliae* on aphids population

A considerable aphids attack was observed at 35 DAT for kale and 40 DAE for okra during both cropping seasons. During the first sampling period, no significant variation in the density of aphids were observed on both crops across the experimental plots except that no sampling was made on apex leaves of kale in the first cropping season. However, application of treatments significantly reduced the population of aphids on okra (Figs 33 and 34) and kales (Figs 35 and 36) in both seasons. There was significant variation ($P <$ 0.05) in aphids' population at 5 days after treatment among treatments in both crops during the two cropping seasons.

In the first cropping season, the percentage reduction in *A. gossypii* population on apex, top and lower leaves of okra plants (i.e., per 20 leaves each) were 80.5, 80.1 and 96.3%, respectively on the plots treated with *M. anisopliae* at 1×10^{12} conidia ha^{-1}. Similarly, application of *M. anisopliae* at 5×10^{12} conidia ha^{-1} resulted in 58.6, 43.0 and 80.4% reduction in *A. gossypii* population on apex, top and lower leaves of okra plants, respectively. The efficacy of Karate® was relatively low (i.e., 28.1, 10.7, 42% on apex, top, and lower leaves. respectively) at 65 DAE (Figs. 33 A, B and C).

In the second cropping season, aphids infestation on okra plots was higher in Karate® treated plots compared to control plots although the variation was non-significant ($P > 0.05$); however there were significant differences between fungus treated and Karate® treated plots, and fungus treated and control plots ($P < 0.05$) at 65 DAE (Figs. 34 A, B and C).

In the first season, the percentage reduction in the population of aphids (*B. brassicae, L. pseudobrassicae* and *M. persicae*) after application of *M. anisopliae* at 1×10^{12} conidia ha^{-1} were 47.6, 56.0 and 47.8% on apex, top and lower leaves of kale plants, respectively, 60 DAT. Similarly, *M. anisopliae* at 5×10^{12} conidia ha^{-1} treatment provided 47.4, 50.4 and 56.9 % reduction in the

population of aphids on apex, top and lower leaves of kale plants, respectively. The standard insecticidal check (Karate®) also resulted in 80.7, 59.8 and 40.6% reduction in aphids population on apex, top and lower leaves, respectively (Figs. 35 A, B and C).

In the second cropping season, there was significant reduction in aphids (*B. brassicae, L. pseudobrassicae and M. persicae*) population on kale plants in the plots treated with the fungus and Karate® compared to the control plots ($P < 0.05$) irrespective of the leaf strata across the sampling periods (Fig. 36 A, B and C). Application of *M. anisopliae* at 1×10^{12} conidia ha^{-1} resulted in 67.1, 61.7 and 58.4% reduction of the three aphids species (*B. brassicae, L. pseudobrassicae and M. persicae*) population on apex, top and lower leaves of kale plants, respectively 60 DAT. Similarly, *M. anisopliae* at 5×10^{12} conidia ha^{-1} treatment resulted 75.3, 71.1 and 63.9% reduction in aphids population on apex, top and lower leaves, respectively. The standard insecticidal check (Karate®) reduced the aphid population by 95.1, 94.5 and 89.0% on apex, top and lower leaves, respectively (Fig. 36). Except at the beginning of the infestation and second season on okra plants, the number of aphids was lower on kales and okra treated with the fungus and Karate® compared to the unprotected controls (Figs 33, 34 and 36).

Mortality caused by application of *M. anisopliae* was observed in all treatments (Tables 13 and 14 A, B and C). During the first season, total mortality of *A. gosspii* varied from 61- 98% while mortality with mycosis varied from 37 to 54% after application of *M. anisopliae* at the rate of 1×10^{12} conidia ha^{-1}. At the application rate of 5×10^{12} conidia ha^{-1}, total mortality varied from 78 - 100 % while mortality with mycosis ranged from 36 - 60% across the spray periods (Table 13). In the second season, total mortality ranged from 81 - 99% and mortality with mycosis ranged from 39- 60% following fungal application at the rate of 1×10^{12} conidia ha^{-1}. Application of *M. anisopliae* at 5×10^{12} conidia ha^{-1} resulted in total mortality ranging from 81 - 98 % while mortality with mycosis varied from 37 - 64 % across the spray periods in okra plots

(Table 13). In the case of kale plots, the total mortality of *B. brassicae* varied from 82 - 96% (21 - 55% mycosed) and 63 - 91% (30 - 60% mycosed) in *L. pseudobrassicae*, in fungus treated plots across spray periods (Tables 14 and15).

In the second season, total mortality in fungus treated kale plots varied from 79 - 99 (25-63% mycosed), 54-94% (23-45% mycosed) and 49 - 88% (9 - 31% mycosed) in *B. brassicae, L. pseudobrassicae* and *M. persicae,* respectively, across the spray periods (Tables 14-16). Mortality due to *M. anisopliae* was also observed in the control plots (Tables 13-16). The variation in terms of total mortality or mycosed aphids due to fungus application was significantly different compared to mortality in the control plots ($P < 0.05$) in both seasons.

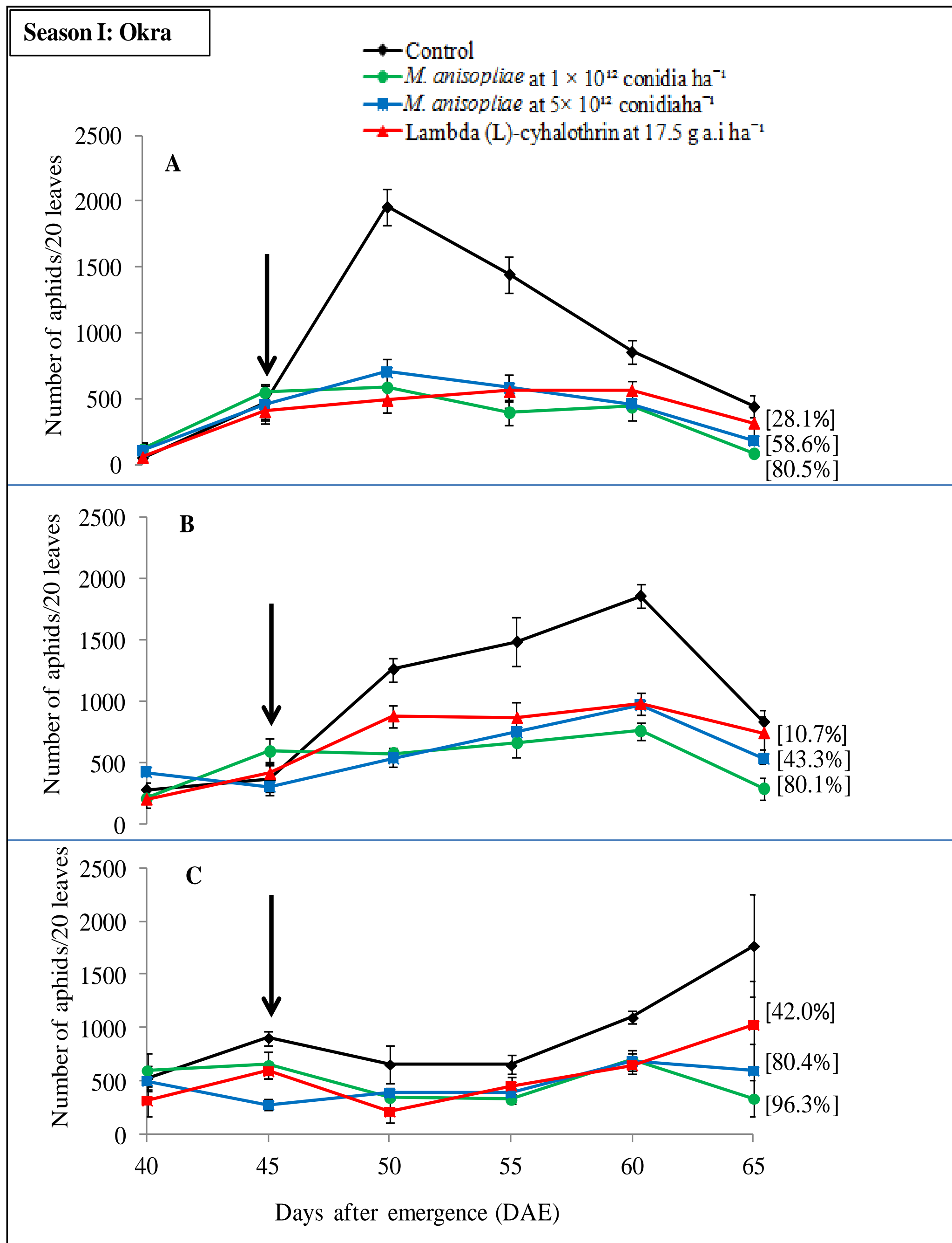

Figure 33. Effect of *M. anisopliae* and lamda (L)-cyhalothrin on the aphids population on okra during the first field trial sampled from three leaf strata: apex leaf (A), top leaf (B) and lower leaf (C) at Mbita, Western Kenya. The arrow denotes the beginning of treatment application. Values in parentheses denote percentage reduction in aphids` density over season.

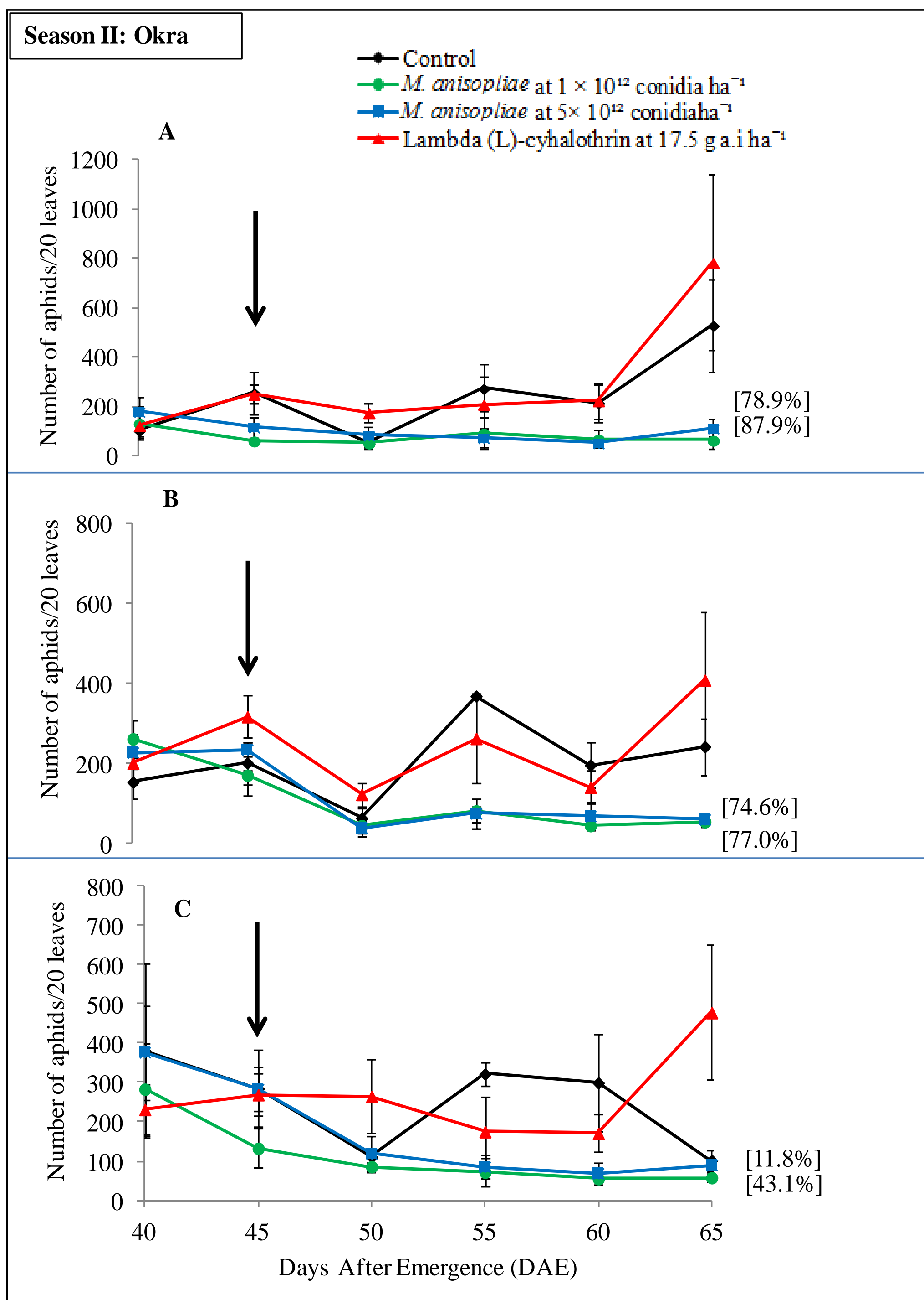

Figure 34. Effect of *M. anisopliae* and lamda (L)-cyhalothrin on the aphids population on okra during the first field trial sampled from three leaf strata: apex leaf (A), top leaf (B) and lower leaf (C) at Mbita, Western Kenya. The arrow denotes the beginning of treatment application. Values in parentheses denote percentage reduction in aphids density over season.

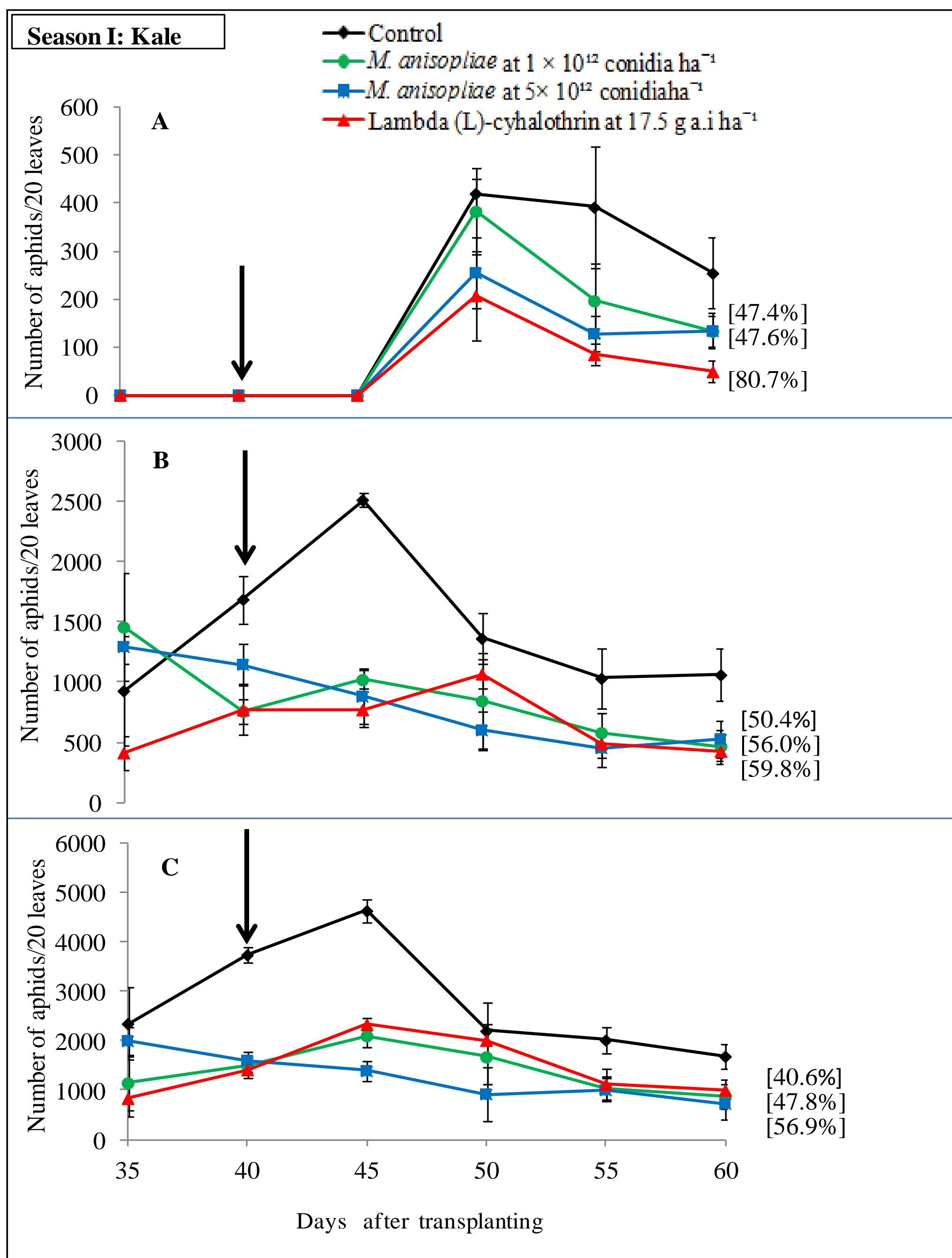

Figure 35. Effect of *M. anisopliae* and lamda(L)-cyhalothrin on the aphids population on kale during the first field trial sampled from three leaf strata: apex leaf (A), top leaf (B) and lower leaf (C) at Mbita, Western Kenya. The arrow denotes the beginning of treatment application. Values in parentheses denote percentage reduction in aphids` density over season.

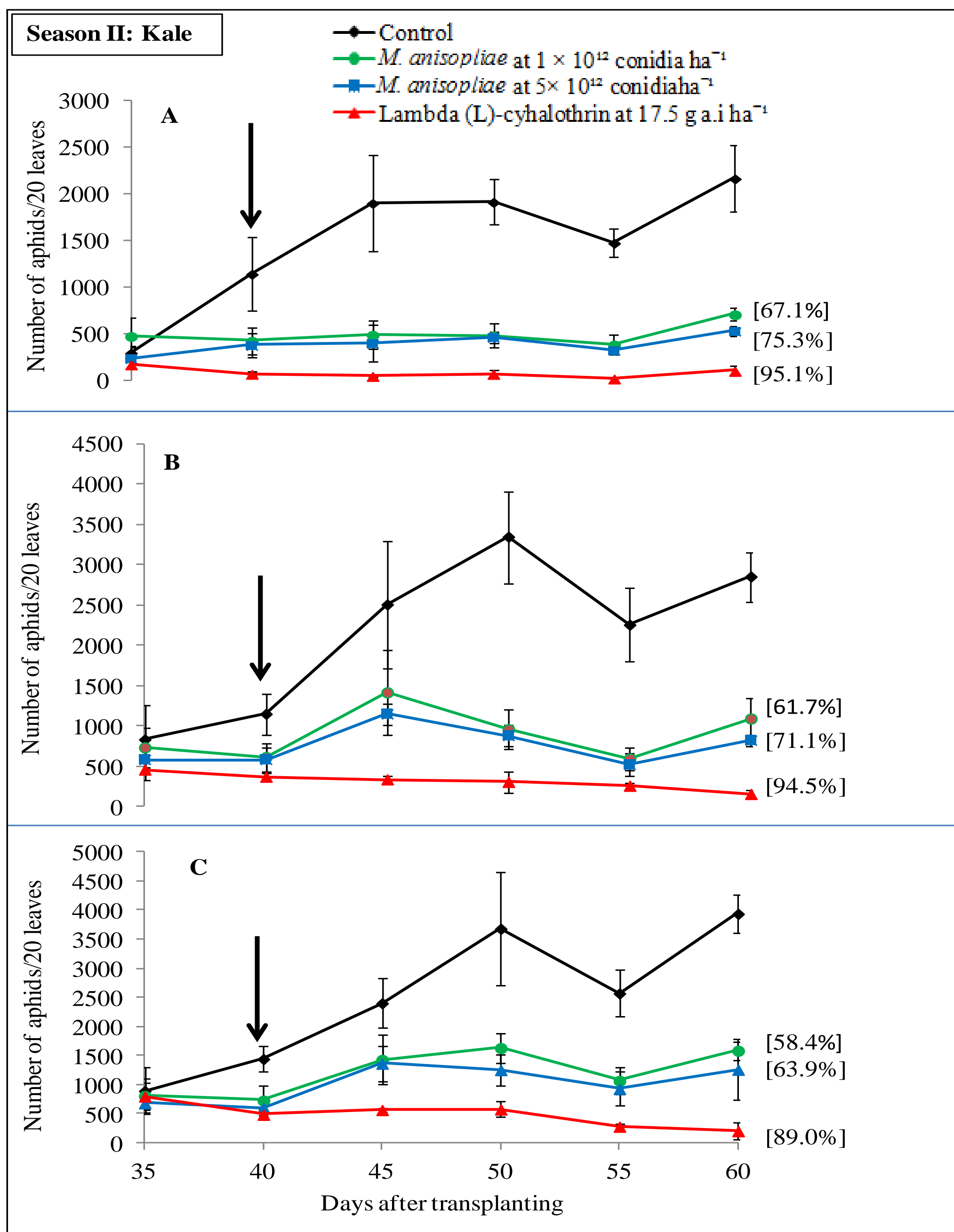

Figure 36. Effect of *M. anisopliae* and lamda (L)-cyhalothrin on the aphids population on kale during the second field trial sampled from three leaf strata: apex leaf (A), top leaf (B) and lower leaf (C) at Mbita, Western Kenya. The arrow denotes the beginning of treatment application. Values in parentheses denote percentage reduction in aphids density over season.

Table 13. Percentage mortality (mean ± SE) in adult *Aphis gossypii* caused by spray application of *Metarhizium anisopliae* on okra plants.

Season/Treatment Mortality	First spray		Second spray		Third spray		Fourth spray		Fifth spray	
	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)
First season										
Control	27.5±3.4b	0.0±0.0b	27.5±4.4b	1.7±1.0b	34.2±2.9b	5.8±2.5b	31.7±2.9b	5.8±1.6b	29.2±2.5b	19.2±3.7c
1 × 10^{12} conidia ha^{-1}	60.8±5.0a	38.3±5.0a	87.5±5.5a	43.3±1.4a	98.3±1.0a	54.2±2.5a	90.8±2.9a	36.7±3.6a	92.5±4.4a	38.3±2.2b
5 × 10^{12} conidia ha^{-1}	77.5±5.5a	35.8±5.0a	81.7±6.7a	40.8±4.4a	100.0±0.0a	50.8±6.0a	95.0±2.2a	44.2±5.5a	89.2±3.9a	60.0±2.4a
Second season										
Control	34.4±4.0b	5.6±1.1b	34.4±2.9b	4.4±1.1b	26.7±8.4b	5.6±2.2b	33.3±3.9b	4.4±1.1b	33.3±5.1b	7.8±1.1b
1 × 10^{12} conidia ha^{-1}	85.6±8.0a	38.9±7.3a	82.2±5.9a	37.8±1.1a	83.3±10.0a	38.9±4.4a	81.1 ±4.0a	42.2±9.1a	98.9 ±1.1a	64.4±6.2a
5 × 10^{12} conidia ha^{-1}	97.8±1.1a	52.2±4.0a	93.3±3.9a	36.7±1.9a	81.1±12.2a	37.8±4.0a	93.3±5.1a	38.9±2.9a	97.8±1.1a	60.0±3.9a

Means (±SE) within columns followed by different letters differ significantly by Student-Newman-Keuls test ($P < 0.05$.

Table 14. Percentage mortality (mean ± SE) in adults of *Brevicoryne brassicae* caused by spray application of *Metarhizium anisopliae* on kale plants.

Season/Treatment Mortality	First spray		Second spray		Third spray		Fourth spray		Fifth spray	
	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)
First season										
Control	26.7±5.9b	0.0±0.0c	35.8±1.6b	5.8±1.6b	33.3±4.1b	5.0±1.0b	27.5±5.7b	5.0±2.9b	37.5±2.1b	8.3±2.2c
1×10^{12} conidia ha^{-1}	95.8±2.5a	45.0±2.3a	91.7±1.0a	31.7±3.5a	90.0±2.4a	32.5±3.7a	81.7±4.8a	31.7±1.0a	89.2±5.7a	35.0±2.2b
5×10^{12} conidia ha^{-1}	86.7±2.4a	20.8±7.5b	85.8±2.5a	34.2±2.9a	85.8±2.5a	38.3±3.5a	87.5±5.5a	32.5±4.2a	93.3±3.6a	55.0±1.0a
Second season										
Control	37.5±2.9b	1.7±0.0c	28.3±8.4b	8.3±2.9b	36.7±1.4b	5.0±2.2c	40.0±3.6b	5.8±2.1b	37.5±5.0b	5.0±2.2b
1×10^{12} conidia ha^{-1}	79.2±7.5a	44.2±6.0a	99.2±0.8a	62.5±7.0a	84.2±2.9a	50.0±3.0a	80.8±6.3a	47.5±7.3a	90.8±3.7a	60.8±6.9a
5×10^{12} conidia ha^{-1}	95.2±1.2a	25.0±3.5b	97.5±1.6a	60.0±12.6a	83.3±3.6a	35.0±3.5b	87.5±4.2a	41.7±3.7a	91.7±1.7a	47.5±2.9a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test ($P < 0.05$.

Table 15. Percentage mortality (mean ± SE) in adults of *Lipaphis pseudobrassicae* caused by spray application of *Metarhizium anisopliae* on kale plants.

Season/Treatment Mortality	First spray		Second spray		Third spray		Fourth spray		Fifth spray	
	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)
First season										
Control	21.7±3.2b	0.0±0.0b	32.5±4.6b	0.0±0.0c	37.5±2.5c	5.8±0.8c	36.7±4.1b	6.7±1.4b	39.2±2.1b	10.8±2.1b
1×10^{12} conidia ha^{-1}	71.7±5.2a	30.0±5.0a	87.5±4.6a	59.2±3.9a	62.5±3.7b	33.3±3.6b	88.3±1.0a	40.8±4.2a	88.3±5.0a	46.7±6.9a
5×10^{12} conidia ha^{-1}	80.8±5.5a	36.7±2.4a	80.0±1.4a	35.0±2.2a	75.8±1.6a	51.7±5.2a	79.2±3.7a	51.7±5.2a	90.8±3.2a	60.0±6.2a
Second season										
Control	33.3±4.7b	1.7±1.7b	37.5±5.0b	10.0±2.4b	23.3±6.5c	1.7±1.0b	33.3±4.1c	1.7±1.0b	38.3±2.9b	6.7±2.4b
1×10^{12} conidia ha^{-1}	71.7±15.5a	30.0±9.9a	90.0±4.5a	37.5±5.0a	70.0±11.9b	23.3±5.9a	54.2±2.1b	24.2±1.6a	74.2±10.1a	45.0±5.5a
5×10^{12} conidia ha^{-1}	76.3±7.0a	36.7±2.4a	94.2±2.5a	30.0±2.4a	92.5±1.6a	35.0±2.9a	68.3±3.2a	26.7±5.6a	72.5±4.8a	35.8±2.1a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test (*P* < 0.05.

Table 16. Percentage mortality (mean ± SE) in adults of *Myzus pesrsicae* caused by spray application of *Metarhizium anisopliae* on kale plants.

Season/Treatment Mortality	First spray		Second spray		Third spray		Fourth spray		Fifth spray	
	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)	Total mortality	Mycosis (%)
Second season										
Control	13.3±3.3b	0.0±0.0c	32.5±4.8b	4.2±0.8b	22.5±2.5b	1.7±1.0b	31.7±3.2b	0.0±0.0b	36.7±3.6b	5.0±2.2b
1×10^{12} conidia ha^{-1}	70.8±8.2a	15.0±4.0b	80.8±3.4a	18.3±2.2a	88.3±4.0a	14.2±6.4a	49.2±7.1a	9.2±2.1a	64.2±4.2a	28.3±4.8a
5×10^{12} conidia ha^{-1}	67.5±5.3a	30.8±2.8a	75.8±12.1a	25.8±3.7a	83.3±5.8a	15.8±1.6a	66.7±6.8a	19.2±4.4a	66.7±2.7a	22.5±6.3a

Means (±SE) within columns followed by different letters differ significantly by Student-Newman-Keuls test ($P < 0.05$).

8.2.3. Flea beetle infestation on okra

The flea beetle count was relatively low at the beginning of the treatments, but increased progressively over time (Fig. 37). Significant variation was observed between Karate® treated and control at 55, 60 and 65 DAE during the first season (P < 0.05); however no significant difference between fungus treated and control plots. During the second cropping season, flea beetle population was also drastically reduced in Karate® treated plots compared to fungus treated and control plots. However, variation was observed between fungus treated and control plots at 60 and 65 DAE.

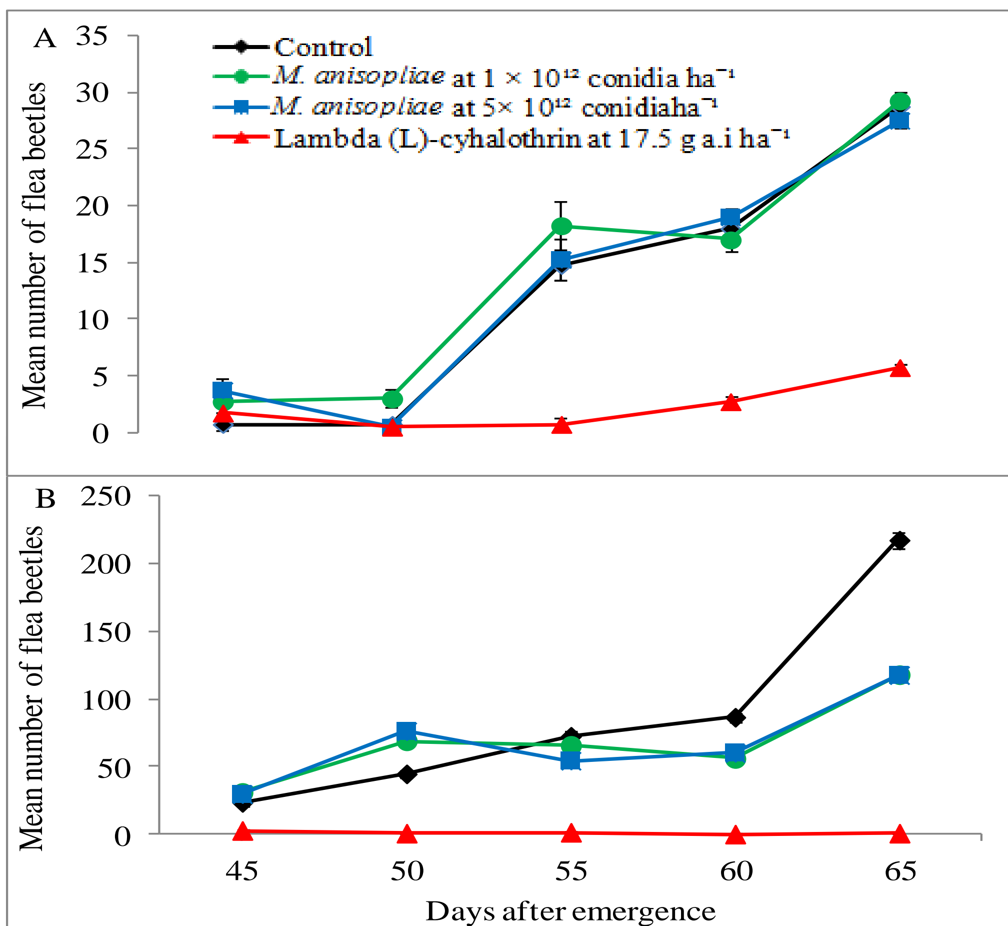

Figure 37. Trends in the mean number (± S.E.) of flea beetles in okra plots treated with *Metarhizium anisopliae* and Karate® during first season (A) and second season (B) field trials.

8.2.4. Okra pod and kale leaf yields

Application of both Karate® and *M. anisopliae* increased marketable yields of okra and kales during the two cropping seasons. Treatments showed significant effect on yield in terms of weight of marketable kale leaves during each cropping season: first season ($F = 4.41$; df = 3, 12; $P < 0.05$) and second season ($F = 1.92$; df = 3, 12; $P < 0.05$) (Table 17). Similarly, there was significant effect of treatment on marketable okra pod yield during the first cropping season ($F = 4.56$; df = 3, 12; $P = 0.02$), but the effect was, non-significant ($P > 0.05$) during the second season (Table 18). Mean separation by SNK showed that marketable yields from fungal treated and Karate® treated plots were not significantly different in both seasons for both okra and kales. On the other hand, fungal treated and Karate® treated plots produced higher yields than the control except for okra in the second season (Tables 17 and 18).

Table 17. Effect of *Metarhizium anisopliae* and Lambda (L)-cyhalothrin application on mean (± SE) marketable kale leaf yield (kg ha^{-1}).

Treatment	First season	Second season
Control	3274.6 ± 203.3b	7621.1 ± 704.8b
M. anisopliae (1×10^{12})*	6831.3 ± 1022.4a	11991.3 ± 92.5a
M. anisopliae (5×10^{12})*	6059.9 ± 1022.4a	12211.8 ± 1220.3a
Lambda (L)-cyhalothrin	6785.3 ± 769.5a	13787.2 ± 1457.5a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test ($P = 0.05$). * Applied concentration of conidia ha^{-1}.

Table 18. Effect of *Metarhizium anisopliae* and Lambda (L)-cyhalothrin application on mean (± SE) okra pod yield (kg ha^{-1}).

Treatment	First season	Second season
Control	17279.3 ± 552.7b	14432.5 ± 1556.0a
M. anisopliae (1 × 10^{12})*	21069.7 ± 1704a	13930.3 ± 1689.0a
M. anisopliae (5× 10^{12})*	23064.0 ± 1614.7a	15633.3 ± 2847.0a
Lambda (L)-cyhalothrin	22504.3 ± 2386.8a	16914.6 ± 671.1a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test (P = 0.05). * Applied concentration of conidia ha^{-1}.

8.2.5. Effect of fungus application on non-target organisms

Application of *M. anisopliae* had no adverse effect on the populations of non-target organisms compared to the Karate® treated plots. Post treatment samples showed that plots treated with Karate® had the most severe adverse effect on the density of non-target organisms when compared to other treatments during both seasons (Tables 19). For instance, population of coccinellids was significantly reduced in Karate® treated kale plots compared to both control plots and fungus treated plots ($P < 0.05$) during the first season.

With the exception of hoverflies and spiders density during the second season, there was no significant difference in the number of non-target organisms recorded before spray application in all okra plots ($P > 0.05$) (Table 20) during both seasons. Similarly, the weekly application of both *M. anisopliae* had no adverse effect on the density of all non-target organisms when compared to the control (Table 20). On the other hand, application of *M. anisopliae* had no adverse effect on the populations of non-target organisms compared to the Karate® treated plots in both crops during both seasons (Tables 19 and 20).

Table 19. Trends in the number of non-target organisms (mean± S.E.) in kale plots treated with *Metarhizium anisopliae* and Karate®.

Treatments/Seasons	Coccinellids		Hoverflies		Spiders		Parasitoids	
	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray
Season one								
Control	5.0 ± 1.3a	12.8 ± 0.4b	13.5 ± 1.4ab	5.0 ± 0.6c	5.3 ± 0.9b	2.8 ± 0.4b	17.8 ± 2.0c	26.9 ± 1.5c
1×10^{12} conidia ha^{-1}	4.3 ± 1.2a	12.7 ± 1.5b	11.0 ± 1.0a	4.0 ± 0.7bc	6.0 ± 1.4b	2.0 ± 0.6ab	7.8 ± 1.1ab	15.3 ± 1.6b
5×10^{12} conidia ha^{-1}	3.5 ± 0.8a	11.2 ± 0.8b	16.0 ± 1.1b	3.3 ± 0.6b	1.0 ± 0.7a	1.9 ± 0.5ab	12.3 ± 1.2b	12.6 ± 1.1a
Lambda (L)-cyhalothrin	1.8 ± 0.8a	0.4 ± 0.2a	16.0 ± 1.3b	0.9 ± 0.5a	3.8 ± 0.8ab	0.7 ± 0.4a	6.5 ± 1.1a	12.6 ± 1.4a
Season two								
Control	3.3 ± 0.8ab	18.1 ± 0.8c	7.0 ± 0.7b	7.6 ± 0.8d	2.5 ± 0.7a	3.3 ± 0.5b	1.5 ± 0.6a	44.7 ± 1.5b
1×10^{12} conidia ha^{-1}	3.3 ± 0.5ab	7.3 ± 0.7b	4.3 ± 0.9ab	4.1 ± 0.7c	5.0 ± 1.1a	2.1 ± 0.5b	0.8 ± 0.6a	65.3 ± 2.8c
5×10^{12} conidia ha^{-1}	1.3 ± 0.5a	10.5 ± 1.1b	2.5 ± 1.0a	2.9 ± 0.5b	2.0 ± 0.5a	1.9 ± 0.6b	0.3 ± 0.4a	68.2 ± 2.4c
Lambda (L)-cyhalothrin	5.3 ± 0.6b	4.8 ± 1.1a	5.3 ± 0.8ab	1.2 ± 0.5a	3.5 ± 1.1a	0.3 ± 0.3a	0.3 ± 0.4a	6.8 ± 0.6a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test ($P < 0.05$) for each season.

Table 20. Trends in the number of non-target organisms (mean± S.E.) in okra plots treated with *Metarhizium anisopliae* and Karate®.

Treatments/Seasons	Lacewing		Coccinellid		Hoverflies		Spiders		Parasitoids	
	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray	Pre-spray	Post-spray
Season one										
Control	2.5 ± 1.0a	1.3 ± 0.4a	3.8 ± 0.9a	2.7 ± 0.8a	1.0 ± 0.3a	2.7 ± 0.4a	1.5 ± 0.6a	2.2 ± 0.6ab	0.0± 0.0a	2.5 ± 0.9a
1×10^{12}conidia ha^{-1}	1.5 ± 0.6a	2.5 ± 0.6a	1.8 ± 0.7a	2.3 ± 0.8a	0.8 ± 0.3a	2.6 ± 0.3a	1.0 ± 0.5a	3.5 ± 0.4b	0.0± 0.0a	2.0 ± 0.9a
5×10^{12}conidia ha^{-1}	2.8 ± 0.6a	2.7 ± 0.5a	3.0 ± 0.9a	2.1 ± 0.3a	1.3 ± 0.4a	1.9 ± 0.3a	1.5 ± 0.5a	2.4 ± 0.5ab	0.0± 0.0a	1.5 ± 0.9a
Lambda (L)-cyhalothrin	2.3 ± 1.0a	1.4 ± 0.6a	2.3 ± 0.9a	1.0 ± 0.4a	3.3 ± 0.4a	1.4 ± 0.3a	1.5 ± 0.7a	0.9 ± 0.4a	0.0± 0.0a	2.3 ± 1.5a
Season two										
Control	0.0 ± 0.0a	0.3 ± 0.3a	2.7 ± 0.4a	2.1 ± 0.5a	3.3 ± 0.3b	0.3 ± 0.3a	1.0 ± 0.7a	0.9 ± 0.3a	0.0 ± 0.0a	0.7 ± 0.4b
1×10^{12}conidia ha^{-1}	0.0 ± 0.0a	0.2 ± 0.1a	2.0 ± 0.8a	1.3 ± 0.5a	0.0 ± 0.0a	0.0 ± 0.0a	1.3 ± 0.6a	1.2 ± 0.4a	0.4 ± 0.3a	0.0 ± 0.0b
5×10^{12}conidia ha^{-1}	0.0 ± 0.0a	0.3 ± 0.3a	3.0 ± 0.8a	1.3 ± 0.3a	0.0 ± 0.0a	0.3 ± 0.3a	4.7 ± 1.0b	2.1 ± 0.6a	0.0 ± 0.0a	0.1 ± 0.2b
Lambda (L)-cyhalothrin	0.0 ± 0.0a	0.0 ± 0.0a	2.3 ± 0.6a	1.7 ± 0.2a	0.0 ± 0.0a	0.1 ± 0.1a	0.7 ± 0.5a	0.7 ± 0.2a	0.4 ± 0.3a	1.7 ± 0.5a

Means (±SE) within each column followed by different letters differ significantly by Student-Newman-Keuls test ($P < 0.05$) for each season.

8.3. Discussion

The results of the various experiments showed that application of aqueous and oil formulations of *M. anisopliae* isolate ICIPE 62 significantly reduced populations of *B. brassicae*, *L. pseudobrassicae* and *A. gossypii* in both the screenhouse and field experiments. In the screenhouse studies, conidia formulated in oil performed better in suppressing (lower ri values) *A. gossypii* population compared to the conidia formulated as aqueous. However, there was no significant difference in terms of this parameter on *B. brassicae* and *L. pseudobrassicae.* Oil alone used as a control had no effect on population growth of all the three aphid species, indicating no toxic effect on the target aphids. This suggests a reduction in population from both adults' mortality and reduction in fecundity following the application of conidial formulations as previously reported by Jandricic *et al.* (2014).

The current study demonstrated that conidia formulated in oil enhance the efficacy of *M. anisopliae* ICIPE 62 against the three aphid species on okra and kale plants. Several studies have also reported similar results on efficacy and speed of kill of oil formulated fungal entomopathogens (Maniania 1993; Ibrahim *et al*., 1999; Ekesi *et al*., 2005; Tumuhaise *et al*., 2015). This is attributed to prevention of conidia from desiccation, increased adhesion and spread of inoculum over the host cuticle and into crevices (Ibrahim *et al*., 1999).

Results of the present study under field conditions showed that *M. anisopliae* isolate ICIPE 62 can provide good control of the aphids on okra and kale, which is comparable to that achieved with Karate®. However, there were variations in aphids' density between the trials. Weather conditions such as rainfall and temperature are important environmental factors that have considerable effect on the rate of development and activities of aphids (Ebert and Cartwright, 1997). Apart from the weather, other factors may have influenced the results of the experiments including (i) difference in aphid genotype (Corletto *et al*., 2010; Chen *et al*., 2012), (ii) difference in population densities of the non-target natural enemies that might have influenced aphid growth through direct attack or

facilitating the transmission of fungus (Roy *et al*., 2001; Ekesi *et al*., 2005; Bayissa *et al*., 2016).

Brevicoryne brassicae and *L. pseudobrassicae* posses long, stilt-like legs and waxed cuticle that could minimize body contact with leaf surface reducing the likelihood of the aphids acquiring lethal dose of inoculum from treated leaf surfaces. . Nevertheless, recent studies by Amnuaykanjaasin et al. (2013) showed that conidia may germinate and penetrate the aphid cuticle most efficiently on the inter-segmental membranes at the proximal end of the legs (close to the body's ventral surface) and this may have contributed to high impact of isolate ICIPE 62 on the highly waxed aphid species.

A high proportion of aphid species collected from the fungus treated plots were mycosed by *M. anisopliae*, which is an indication of the effects of fungal treatments. However, few mycosed insects were also observed from control plots in both seasons indicating the migration of infected insects among other factors. This corroborates the findings of Ekesi *et al*. (1999) who reported mycoses of *Megalurothrips sjostedti* (Trybom) from infection of *M. anisopliae* in untreated cowpea plots.

Application of Karate® drastically reduced flea beetle populations in okra plots during both seasons and was reflected in pod yield although not significant. Despite the broad host range of *M. anisopliae*, ICIPE 62 has not shown adverse effect on flea beetle population on okra plants. This supports the assertion that certain strains and genotypes of *M. anisopliae* are more effective to particular host species (Bidochka and Small, 2005).

Application of *M. anisopliae* at 1×10^{12} conidia ha^{-1} and 5×10^{12} conidia ha^{-1}, and Karate® significantly increased kale leaf yields in both cropping seasons and okra pod yields during the first season. In the second season, okra pod yields from fungus and Karate® treated plots were not significantly different from the control plots. The high number of flea beetle during this season might have contributed to the reduction in okra pod yields as indicated in Fig.41.

Fungal biopesticides continue to gain importance as alternative control agents against a variety of insect pests either as a stand-alone control agents or as a component of IPM. Thus, assessment of EPF on non-target organisms is an important criteria in the development of mycoinsecticides (Moore and Prior, 1993; Ekesi *et al*., 1999; Maniania *et al*., 2003; Bayissa *et al*., 2016). The result of the current study showed no significant detrimental effect of *M. anisopliae* on non-target organisms compared with the controls. This result is in agreement with the laboratory studies (Chapter 5 and 6). Similar results were also reported by Ekesi *et al*. (1999) and Maniania *et al*. (2003) in cowpea and onion cropping systems, respectively. Furthermore, some isolates of EPF pose minimum risk to non-target arthropods under field condition (Goettel *et al*., 1990) and more specific compared to laboratory studies (Jaronski *et al*., 2003). The generalist coccinellid predators and aphid specific parasitoids recorded in these studies confirm their compatibility with *M. anisopliae* ICIPE 62 as observed in the laboratory studies (Chapter 5 and 6). The various taxa and species of predators and aphid specific parasitoids collected in this study can be incorporated with *M. anisopliae* in integrated management package for the aphid pests on crucifers and okra.

8.4. Conclusions

The present study demonstrates that *M. anisopliae* isolate ICIPE 62 holds great potential as a biopesticide for managing aphids' species on okra and crucifer plants. In addition, since aphids usually form large colonies on plant surfaces, the *M. anisopliae* isolate ICIPE 62 wich sporulates profusely on dead aphids could serve as source of inoculum for infection of healthy aphids which may lead to acute mortality. Some of the non-target organisms recorded in the present study are known for suppressing aphids which suggests that the use of *M. anisopliae* would be compatible with the potential natural enemies for aphid management on okra and crucifer. This should reduce dependence on broad spectrum synthetic insecticides, promote biodiversity conservation and environmental quality. However, further studies need to be explored on the effect of host plant varieties and different cropping pattern on efficacy of the fungus.

CHAPTER NINE: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

9.1. General discussion

The use of EPF as a biopesticide can offer an environmentally benign alternative for aphid management. Among many taxa and species, *B. bassiana* and *M. anisopliae* are ubiquitous in nature and have been widely investigated against diverse invertebrate pests including aphids. Identifying virulent isolates of EPF that are effective against a key vegetable-infesting aphid species is an important step towards the development of successful IPM package for these pests. Fungal strains with rapid kill, mass production potential and formulation type are among important parameters required for selecting candidate isolate(s) for biopesticide development (Butt and Goettel, 2000; Wraight *et al.*, 2007; Vega *et al.*, 2012). The development of existing strains in a region of use is also more suitable than introduction of exotic strains due to ecological and political concerns (Bidochka, 2001). The current study was, therefore, sought to identify novel isolate(s) with high levels of virulence against *B. brassicae, L. pseudobrassicae* and *A. gossypii* and develop them for the management of the target pests.

Results from this study showed that all the isolates of *B. bassiana* and *M. anisopliae* tested were pathogenic to *B. brassicae, L. pseudobrassicae* and *A. gossypii.* There was, however, significant variation in the pathogenicity between species and isolates within species. *Metarhizium anisopliae* isolates were generally more pathogenic than *B. bassiana*. Of the *M. anisopliae,* three of them (ICIPE 30, ICIPE 62 and ICIPE 69) induced high mortality and required short time to kill 50% of all the three aphid species. Thus, these three isolates were selected for dose-response study, conidial yield and thermotolerance studies.

Dose-mortality relationship revealed that three isolates of *M. anisopliae* isolate ICIPE 62 outperformed the other fungal isolates. Of the three isolates, ICIPE 62 required low median lethal concentration of conidia to kill 50% of all the three aphid species. The degree of pathogennicity among fungal species and isolates of the same fungal species are expected during the routine screening tests against various arthropod pests. For example, isolartes of *B. bassiana* and *M. anisopliae*

exhibit variability in pathogenicity against crucifer pests, *Phaedon cochleariae* Fabricius**,** *M. persicae* and *Lipaphis* erysimi Kaltenbach which *M. anisopliae* were most pathogenic (Butt *et al*., 1994). Isolate ICIPE 62, identified in this study as being highly pathogenic to the three aphid species has also been reported to be virulent against *Ceratitis rosa* var. *fasciventris* (Karsch), and *C. cosyra* (Walker) (Diptera: Tephritidae) (Dimbi et al. 2003) and adult *Cylas puncticollis* Boheman (Coleoptera: Curculionidae) (Ondiaka et al. 2008). In addition to virulence, the conidation potential of isolate ICIPE 62 on aphid cadavers can also increase the spread of infection among aphid populations. This suggests some EPF genere could be host-specific, but can have isolates that can be effecious against wide host range. It is, therefore, important to investigate the possible nontarget effect of candidate biopesticide.

Environmental factors including temperature are known to influence the effectiveness of EPF (Fargues et al., 1997). In developing fungal-based biopesticide for the management of aphids, it is important to evaluate the efficacy of candidate isolates at temperatures range that may be limiting in the field. Results of the present study demonstrated that *in vitro* germination and growth of the three selected isolates of *M. anisopliae* were noticeably reduced at 10, 15 and 35ºC. However, maximum germination and growth was achieved between 20 - 30ºC. In addition to *in vitro* germination and growth, optimum temperatures for virulence of all the three isolates to the aphid species was achieved at 25 and 30°C. Of the three isolates, thermotolerance ability of ICIPE 62 matched the environment in which it is to be used as a biopesticide. A correlation between optimum temperature for fungal growth and fungal infection observed in this study has also been reported by different authors (Maniania and Fargues, 1992; Ekesi *et al*., 1999; Bugeme *et al.,* 2008). This suggests that the insects would be susceptible to fungal infection at the temperatures at which the three aphid pests are active in field, and it should be suitable in the tropical regions.

As a step towards the development of biopesticide, it is also necessary to evaluate the compatibility of EPF candidate isolate with non-target organism if they have to be used together within the context of aphid IPM programs (Jaronski *et al*., 1998). The present study revealed that *M. anisopliae* ICIPE 62 has minimal

adverse effect on the survival of the predatory coccinellids, *C. lunata*, causing mortality of less than 10%; which supports the assertion that despite the wide host range of *M. anisopliae*, certain isolates and genotypes of EPF are more limited to particular species of insects (Bidochka and Small, 2005). Host specificity of the isolate should permit application of this isolate with other natural enemies.

The study also demonstrated that neither starved nor non-starved adult female *C. lunata* showed feeding preferences towards fungus-infected aphids. On the other hand, the study revealed that the predatory coccinellid (*C. lunata*) served as a means for transmission of infection to uninfected aphids on host plants by first coming into contact with fungus-infected ones. This suggest that combined use of the two agents, therefore, complement one another in the suppression of aphids.

The lethal and sub-lethal concentrations of *M. anisopliae* ICIPE 62 had no pronounced adverse effect on parasitism rate, emergence, sex ratio, longevity and morphometry of *A. colemani* and *D. rapae.* For example, longevity of F_1 generation females and males of *D. rapae* and *A. colemani* that emerged from host aphids inoculated with ICIPE 62 were not significantly different from the control. On the other hand, high proportion of mummies were prodiuce when host aphids were first exposed to parasitoids and then infected by fungus. However, such variation was not observed when aphid first exposed to parasitoid and then treated by fungus. Variation in parasitism rate (production of mummies) suggests that time of application of fungus is important if the two agents will be used together to control aphids. Generally, the various findings in this study demonstrate the possibility of combined use of ICIPE 62 and the two parasitoid species in the management of aphids, which is a huge advantage as compared to synthethic chemical insecticides.

Application of aqueous and oil formulated conidia of *M. anisopliae* isolate ICIPE 62 significantly reduced population of *B. brassicae, L. pseudobrassicae* and *A. gossypii* on okra and kale plants in the screenhouse condition. The present study also established that oil formulated *M. anisopliae* was effective in suppressing aphid populations, resulting in high mortality of adults of *B. brassicae. L. pseudobrassica*e and *A. gossypii*. Previous studies have demonstrated that oil formulation increases infection by spreading of inoculum over the host cuticle

surfaces and into crevices (Ibrahim *et al*., 1999; Gindin *et al*., 2001). Use of oil formulation is important to increasing moisture on insect cuticle as it wets the hydrophobic and lipophilic surfaces of insects (Jenkins and Goettel, 1997). As a result, it increases efficacy of the EPF by improving adhesion of conidia to insect cuticle as well as provide protection against adverse environmental factors (Moore and Prior 1993). Wraight *et al*. (2001) and Sedighi *et al.* (2013) demonstrated that oil is a superior spray carrier for its inherent compatibility with lipophilic conidia of EPF.

Studies from the two cropping seasons under field conditions showed that ICIPE 62 and chemical pesticide (Karate®) significantly reduced aphid populations in both okra and kale plots. However, increasing population growth of *A. gossypii* was observed 60 DAE in chemical treated okra plots. ICIPE 62 was as effective as Karate® against aphids on kale plants and in some instances better against *A. gossypii* during the second cropping season on okra. Karate® was highly effective against flea beetles. The control plots recorded the highest population of aphids on both crops during the two cropping seasons.

A high proportion of aphid species collected from the fungus treated plants both under screenhouse and open field were mycotized by *M. anisopliae,* which is an indication of the effects of fungal treatments.

Furthermore, present field study confirmed the compatibility of *M. anisopliae* isolate ICIPE 62 with important natural enemies that were observed during the laboratory studies. Most of these natural enemies are generalists, but their use together with the candidate biopesticide (ICIPE 62) can be considered for the management of aphid pests in okra and kale cropping system.

9.2. General conclusions

1. Among the tested isolates, *M. anisopliae* isolate ICIPE 62 is the most promising candidate biopesticide for the control of *B. brassicae, L. pseudobrassicae* and *A. gossypii* on crucifer and okra plants.

2. *Metarhizium anisopliae* isolate ICIPE 62 was compatible with arthropod natural enemies, especially the generalist predator *C. lunata* and aphid specific parasitoids*, A. colemani* and *D. rapae.*
3. Presence of *C. lunata* on host plants enhanced transmission and spread of conidia to healthy aphids.
4. Oil formulation of conidia enhanced the efficacy of ICIPE 62 against *B. brassicae, L. pseudobrassicae* and *A. gossypii* on crucifer and okra.
5. Application of ICIPE 62 reduced aphid populations which resulted in kale and okra yield increments that was comparable to those achieved with the commonly used synthetic insecticide.
6. ICIPE 62 has minimal effect on non-target organisms and is therefore compatible with other natural biological approaches necessary for IPM. Hence, promote conservation of arthropod biodiversity.

9.3. Recommendations and future works

1. *Metarhizium anisopliae* isolate ICIPE 62 is highly virulent to adults of *B. brassicae, L. pseudobrssicae* and *A. gossypii.* It is also important to determine the effectiveness of the isolate against the various nymphal stages of aphids.
2. Oil formulation of *M. anisopliae* isolate ICIPE 62 is recommended as an effective biopesticide for the suppression of the aphids populations.
3. Based on the laboratory and field observations, *M. anisopliae* isolate ICIPE 62 is compatible with *C. lunata* for the management of *B. brassicae, L. pseudobrssicae* and *A. gossypii*, and recommended to be utilized as a component of IPM on crucifer and okra. Further studies should generate information on the effect of *M. anisopliae* on female *C. lunata* fecundity and fungus derived volatiles from sporulating cadaver on foraging behaviour.
4. To more fully understand the interactions that occur between *M. anisopliae* ICIPE 62 and *D. rapae* and/or *A. colemani*, fungus-infected host acceptability and suitability by the parasitoid, effect on the different developmental stages of the parasitoids and reproduction potential of F_1 female parasitoid require further investigation.
5. *Metarhizium anisopliae* ICIPE 62 is a suitable candidate biopesticide for the management of *B. brassicae, L. pseudobrssicae* and *A. gossypii*; and oil

formulation of the fungus at 1×10^{12} conidia ha^{-1} with appropriate timing of spray application should facilitate efficient use under field conditions.

6. Studies addressing multitrophic interactions among host plants, predators, parasitoids and the fungal pathogen under different cropping patterns (e.g. monoculture, inter-cropping) should generate further information on the role of the biopesticides on the population dynamics of aphid pests and their natural enemies.
7. Since different agrochemicals are used for the management of other pests of crucifer and okra, compatibility of these chemical insecticides with ICIPE 62 should be undertaken.
8. Aphid-borne diseases such as viruses are becoming important in terms of crop damage. Future studies should establish whether infection by entomopathogenic fungi in aphids can impact on virus transmission.

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Annex

Annex 1. Effects of *M. anisopliae* (oil and aqueous formulated) sprayed-okra (A) and kale (B) plants on aphids in the screenhouse at *icipe*.

A) Aphid infested/attacked on water and oil sprayed control and healthy/less-attacked on aqueous and oil formulated conidia of *M. anisopliae* sprayed-okra seedlings in the screenhouse.

B) Aphid infested/attacked on water and oil sprayed control and healthy/less-attacked on aqueous and oil formulated conidia of *M. anisopliae* sprayed-kale seedlings in the screenhouse.