EVALUATION OF ENTOMOPATHOGENIC FUNGAL ISOLATES FOR MANAGEMENT OF *RHOPALOSIPHUM PADI* AND *METOPOLOPHIUM DIRHODUM* IN WHEAT *(TRITICUM AESTIVUM)*

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A thesis submitted to the Graduate School in fulfillment of the requirements of the Doctor of Philosophy Degree in Crop Protection of Egerton University

Egerton University

MARCH 2015

DECLARATION AND APPROVAL

DECLARATION

This thesis is my original work and has never been presented in this or any other university for the award of a diploma or a degree.

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DEDICATION

To my mother, Susan, who in a myriad ways has been a source of tremendous encouragement and inspiration to me throughout my life and actively supported me in my determination to find and realize my potential, and to make this contribution to our world.

ACKNOWLEDGEMENTS

Through this acknowledgment, I express my sincere gratitude to all those who have supported, encouraged, mentored, and helped as I have traversed the hilly path to completing this thesis. First of all, I thank the Almighty God for his faithfulness and amazing grace that made what at some point appeared impossible possible. I salute Egerton University through Crops, Horticulture and Soils Department for the continued support of, and interest in this research. The achievement of this study would not have been realized were it not for the thorough supervision and expert advice from, Prof. Alice W. Kamau, a seasoned entomologist from the Crops, Horticulture and Soils Department of Egerton University, Prof. Peter F. Arama, an established mycologist from Rongo University and Dr. Nguya K. Maniania, an experienced insect Pathologist from Arthropod Pathology Unit, *icipe*. My heart-felt gratitude to Prof. Alice Kamau who engaged me consistently in making sense of the various challenges I faced and provided not only academic advice but also moral and spiritual encouragement at those times when it seemed impossible to continue. She lovingly challenged and supported me throughout this work - knowing when to push and when to let up. During my extended period of study Prof. P. F. Arama became both a mentor and a friend, helping me get 'unstuck' with this thesis on many occasions. It is with great gratitude that I recognize him as both the inspiration for this work and the means of completing it. Like most influential men, he is supported by a great woman, who created space, oftentimes at short notice, and extended great hospitality whenever I visited their home for consultations. I am deeply indebted to Dr. N. K. Maniania for providing valuable conceptual insight and guidance for virulence bioassays and allowing me to use facilities in his laboratory. The pervasive friendship and cooperation that abounded among the cohort of scientists and laboratory technicians made this work truly enjoyable. Special thanks to Ms. Elizabeth Ouna for patiently conveying her superior laboratory skills to me as I began my graduate work. My heart-felt gratitude to Joseph Mutisya for tremendous research assistance in laboratory and greenhouse experiments. I am also grateful to Mr. Joseph Macharia, the Principle Technologist at Egerton University research fields for assistance in field trials. I thank Mr. John Kamundia of KALRO, Njoro for his vast statistical experience, enthusiasm, and willingness to patiently instruct and help me with the statistical analyses of this research. I am most appreciative of the German Academic Exchange Service (DAAD), for financial support in the initial stages of this research. I am also supremely grateful for the support and encouragement I have received from my family. Special thanks to my beloved mum Susan and beautiful daughters Mitchel, Maisie, Tamara and son Trevor who patiently endured my absence during the strain of thesis research and writing. It is a reassuring anchor to know that the ones I love sustain my professional pursuits.

ABSTRACT

The success of entomopathogenic fungi as microbial control agents ultimately depends on the use of the right propagule, formulated in an optimum fashion and applied at the right time to a susceptible host. In this study, the pathogenicity of six isolates of *Beauveria bassiana* (Bals.) Vuill. and 14 isolates of *Metarhizium anisopliae* (Metsch.) Sorok to *Metopolophium dirhodum* (Walker) *and Rhopalosiphum padi* (Linneaus) was for the first time determined in the laboratory. The differential susceptibility of different developmental stages to fungal infection and the effect of different fungal formulations on aphid mortality and aphid population growth attributes was also established. Time-mortality (LT_{50}) laboratory study results showed that three out of the twenty screened isolates, identifiable as ICIPE 84, ICIPE 51 and ICIPE 23 registered significantly ($P< 0.05$) shorter periods of lethal infection, indicating high level of pathogenicity or virulence of the select isolates. Further dose-response bioassays indicated that ICIPE 51 outperformed isolates ICIPE 23 and ICIPE 84 by recording the lowest LC_{50} and LC_{90} values. Virulence for all nymphal stages was dose-dependent and mortality increased with host aphids' maturity and over time. Low doses of the isolate did not affect pre-lethal reproductive effects, such as fecundity and intrinsic rate of increase. Both aphid species were significantly ($P< 0.05$) more fecund in their early adulthood, suggesting the stage as ideal for biopesticide management intervention. Greenhouse and field trials established that *M. anisopliae* isolate ICIPE 51 has a great potential for management of *R. padi and M. dirhodum*, particularly when formulated in oil. However, if the entomopathogen is to be applied prophylactically, before infestation of wheat with aphids, then a sticker-based formulation would be most preferable. Additional work is required to further critically evaluate the bio-efficacy of the virulent strains in field conditions, examine methods for mass production and long term preservation as well as explore possibilities of integrating the use of the fungal isolates with other control tactics as a component of Integrated Pest Management (IPM) programmes.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis Of Variance
APU	Arthropod Pathology Unit
ARPPIS	African Regional Program in Insect Science
BCAs	Biological Control Agents
BYDV	*Barley yellow dwarf virus*
CHS	Crops, Horticulture and Soils Department
CV	Coefficient of Variation
DAAD	German Academic Exchange Service
DAT	Days After Treatment
EIL	Economic Injury Level
EPF	Entomopathogenic Fungi
FAO	Food and Agriculture Organization (of the United Nations)
GOK	Government of Kenya
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
KALRO	Kenya Agricultural and Livestock Research Organization
LC_{50}	Median Lethal Concentration
LSD	Least Significant Difference
LT_{50}	Median Lethal Time
MD	*Metopolophium dirhodum*
POPs	Persistent Organic Pollutants
RP	*Rhopalosiphum padi*
SAS	Statistical Analysis for Sciences
SDA	Sabouraud Dextrose Agar

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Wheat, a cereal grass of the Gramineae (Poaceae) family and of the genus *Triticum* and its edible grain, is the world's largest cereal-grass crop. One reason for its popularity is that, unlike other cereals, wheat contains a high amount of gluten, the protein that provides the elasticity necessary for excellent bread making (Kamau, 2013).

Although over 30,000 varieties of wheat exist, the two major types are bread wheat and durum wheat. Hard wheat is high in protein (10 to 17 percent) and yields a flour rich in gluten, making it particularly suitable for yeast breads. The low-protein (6 to 10 percent) softer type yields flour lower in gluten and, therefore, better suited for tender baked products, such as biscuits, pastries, and cakes. Although most wheat is grown for human food, industry uses small quantities to produce starch, paste, malt, dextrose, gluten, alcohol, and other products. Inferior and surplus wheats and various milling byproducts are used for livestock feeds. Wheat grain is a major source of energy in human diet and its caloric content is greater than of any other food crop. Wheat is also used as an ingredient in compound feedstuffs, starch production and as a feed stock in ethanol production (FAO, 2014)

On an average the kernel contains 12 percent water, 70 percent carbohydrates, 12 percent protein, 2 percent fat, 1.8 percent minerals, and 2.2 percent crude fibers. Thiamine, riboflavin, niacin, and small amounts of vitamin A are also present. A half kilogram of wheat contains about 1,500 calories. In West Asia/North Africa, as well as Central Asia, it contributes more calories to diets than all other cereals combined (Kamau, 2013).

Wheat is the second most important cereal crop in Kenya after maize (GOK, 1997), with current national wheat production at nearly 300,000 tons, which is 60% less than the national demand of about 700,000 metric tons (Mukisira *et al.,* 2012; FAO, 2014). This shortfall has led to wheat imports of 70% annually to meet the demand (Kinyua *et al.,* 2003). The high increase in population and consumption of wheat products is expected to have a bigger

impact on wheat production and importation in future with the demand reaching 850,000 tons in 2020. Boosting wheat production requires high-yielding crop varieties, effective pest control measures, increased soil fertility and better soil and water management (Kamau, 2013).

Among the pests of economic importance in wheat in Kenya are cereal aphids (Wanjama and Arama, 1999; Wangai *et al.*, 2000) which also occur and cause severe damage to the crop worldwide (Blackman and Eastop, 2007). Different cereal aphid species occur on wheat fields causing serious yield losses through their direct and indirect damage (Ehsan-ul-Haq, 2003; Nyaanga *et al*., 2006; Van Emden and Harrington, 2007; Lapierre and Hariri, 2008). The rose-grain aphid *Metopolophium dirhodum* (wlk.), along with the bird cherry-oat Aphid, *Rhopalosiphum padi* (Linneaus), are two of the species most frequently found in Kenya. Although these aphids can remove considerable amounts of liquid and nutrients from phloem, and strong infestations can sometimes lead to leaf contortion, the direct effect on grain yield is generally minor, especially if plants are infested when they are young. The insects cause the most damage by transmitting a number of viruses, especially *Barley yellow dwarf virus* (BYDV), for which they are most important vectors (Riedell *et al*., 2003; Jiménez-Martínez *et al*., 2004; Fabre *et al*., 2006; Borer *et al*., 2009 and A. Wangai, personal communication). Aphid-transmitted viruses cause diseases of major economic importance in crops (Hull, 2002) such as cereals (Plumb, 2002).

Various control methods including cultural, chemical, biological and host plant resistance have been used with varying degrees of success. Cultural practices include the manipulation in time of planting. Early planting may reduce aphid build up but this is only useful if environmental conditions do not promote aphid build up as occurs during dry periods. Insecticide use and particularly contact foliar applications are ineffective because of the feeding nature of the aphids. The aphids feed within the rolled leaf whorl so they cannot be easily reached by contact foliar sprays. Reliance on chemical control is expensive and presents a danger of production of resistant biotypes, destruction of biological agents and has a bearing on the quality of environment. A long-term control strategy such as the use of

biological agents is preferred because it is more effective and sustainable although not yet immediately available. (Dutcher et al., 2003)

In order to optimize the environment-agriculture sustainable development relationship, scientific and technological knowledge regarding the modernization of pest control management needs complex research approaches in a systematic, agro-ecologically integrated manner (Popov *et al.*, 2009; Malschi *et al*., 2012). Entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales) offer an environmentally friendly alternative to chemical pesticides. These fungi are natural, can easily be formulated, are less toxic to mammals, leave no harmful residues (Copping, 2004) and problems with resistance are less likely to occur (Zimmermann, 2007a, b). *Metarhizium anisopliae* and *Beauveria bassiana* have a wide host range, are widely distributed in all regions of the world and can be easily isolated from insects, soil and phylloplanes of hedgerow vegetation (Meyling and Eilenberg, 2006). Several products based on strains of *Lecanicillium* sp. (Jung *et al*., 2006), *B. bassiana, M. anisopliae* and *Paecilomyces* have been reported to control a range of pests including aphids and whiteflies (Yeo *et al*., 2003 and Bruck, 2005). Today, biological control has become a key component of crop protection worldwide and relies on the introduction of exotic or naturally occurring biological control agents (BCAs) which permanently suppress pests and persist in the environment.

1.2 Statement of the problem

Different cereal aphid species occur on wheat fields causing serious yield losses through their direct and indirect damage. Demand for wheat and wheat-based products cannot be met by domestic production. Domestic production only meets a third of her national wheat requirements with imports filling two-thirds. Population growth and growing preference of wheat products as a convenient food, largely accounts for the increase in wheat demand. Kenya's wheat imports grew at an annual average rate of nine percent between 2006 and 2011 calendar years. Consequently, forecasts indicate that wheat imports will continue growing, exceeding one million metric tons annually. A significant constraint to meeting

that growing demand in wheat production is the reduction of yield by direct and indirect damage of feeding activities of aphids.

A major problem facing the bread wheat industry in Kenya is that most commercial varieties are susceptible to *Barley yellow dwarf virus* transmitted by *Rhopalosiphum padi* and *Metopolophium dirhodum*. Aphicide use in Kenya against these pests has risen, but farmers report that even with their use, only 60% of crop yields are realized. Some farmers have even attempted to use aphicides beyond the recommended levels to save their crop. In addition to their indirect involvement as virus vectors, aphids have large reproductive rates and their wide range of host plants mean that they are difficult to control. Selective aphicides have been used to reduce populations below damage threshold levels. If they are to be used as sole control agents for pests that have several generations during a crop cycle, they will have to be applied repeatedly because of the high reproductive capacity of the pest.

Intensive crop production and extensive use of harmful synthetic chemical pesticides creates numerous socio-economic problems worldwide. Probably the most profound is the harm the pesticides are doing to humans and the environment. In addition, there are increasing reports of pesticide resistance in pest populations. In the long term, exports of Kenyan produce could also be affected especially to countries which have stringent measures to monitor residues on produce. There is therefore an urgent need to develop more benign agents and strategies for the control of crop pests.

1.3 Justification

The rose-grain aphid *Metopolophium dirhodum* (wlk.), along with the bird cherry-oat aphid, *Rhopalosiphum padi* (Linneaus), are two of the species most frequently found in Kenya. They cause direct damage by reduction of grain yield and indirect damage by transmission of a number of viruses, especially *Barley yellow dwarf virus* (BYDV), for which the two species are the most important vectors (Riedell *et al*., 2003; Jiménez-Martínez *et al*., 2004; Fabre *et al*., 2006; Borer *et al*., 2009; A. Wangai, personal communication).

A major problem facing the bread wheat industry in Kenya is that most commercial varieties are susceptible to *R. Padi* and *M. dirhodum*. Chemical pesticides can lead to pesticide resistance by the aphids and/or the destruction of beneficial natural enemies (Dutcher et al., 2003). Many of the chemical insecticides currently used to control aphids are listed among the most persistent organic pollutants (POPs) by the United Nations Environmental programme (UNEP). There is therefore an urgent need to develop more benign agents and strategies for the control of crop pests.

Among the different microbial agents, entomopathogenic fungi (EPF) are gaining importance in pest control. More than 750 species of fungi are pathogenic to insects and many of them offer a great potential for the management of sucking pests (Rabindra and Ramanujam, 2007). *Metarhizium anisopliae* and *Beauveria bassiana* have been studied and applied to manage various insect pests on many crops around the world (Butt and Copping 2000; Goettel *et al*. 2000; Nugroho and Ibrahim 2007). The fungi are highly specific to hosts. At field application rates, they are considered safe to non–target hosts. The fungi can be mass-produced relatively easily on artificial solid substrates and when formulated in oil, can be applied under a wide range of environmental conditions using commonly available pesticide application equipment (Dhaliwal and Koul, 2007). Considering the adverse effect of insecticides, research toward developing alternative pest management tactics is clearly warranted.

1.4 Objectives

1.4.1 Broad Objective

To increase wheat production through sustainable management of cereal aphid vectors of *Barley yellow dwarf virus (*BYDV).

1.4.2 Specific Objectives

The specific objectives were to:

i) Determine pathogenicity of different *Metarhizium anisopliae* (Metch) and *Beauveria Bassiana* (S) isolates to *Rhopalosiphum padi* (L) and *Metopolophium dirhodum* (Walker)

ii) Identify the most efficacious isolates from dose-response effects of selected *Metarhizium anisopliae* (Metch) isolates to *Rhopalosiphum padi* (L) and *Metopolophium dirhodum* (Walker)

iii) Investigate the differential susceptibility of various developmental stages of *Rhopalosiphum padi* (L.) and *Metopolophium dirhodum* (Walker) to infection by *Metarhizium anisopliae* (Metch) isolate ICIPE 51

iv) Determine the effects of *Metarhizium anisopliae* (Metch) isolate ICIPE 51 infection on fecundity and intrinsic rate of increase of *R. padi* and *M. dirhodum*

v) Determine the efficacy of three formulations of *M. anisopliae* isolate ICIPE 51 (oil, sticker and water) against *R. padi* and *M. dirhodum* when applied before (Pre) and after (Post) infestation of wheat

1.5 Hypotheses

The following null hypotheses were developed and tested:

i) There is no difference in the level of pathogenicity of different *Metarhizium anisopliae* (Metch) and *Beauveria Bassiana* (S) isolates to *Rhopalosiphum padi* (L) and *Metopolophium dirhodum* (Walker)

ii) There is no difference in the LD_{50} and lD_{90} values of selected three EPF isolates

iii) There is no differential susceptibility of various developmental stages of *Rhopalosiphum padi* (L.) and *Metopolophium dirhodum* (Walker) to *Metarhizium anisopliae* (Metch) isolate ICIPE 51 infection

iv) There is no differential fecundity and intrinsic rate of increase of *R. padi* and *M. dirhodum* due to infection with different formulations of *M. anisopliae* isolate ICIPE 51

v) There is no variation in efficacy of three formulations of *M. anisopliae* isolate ICIPE 51 (oil, sticker and water) against *R. padi* and *M. dirhodum* when applied before (Pre) and after (Post) infestation of wheat

CHAPTER TWO
LITERATURE REVIEW

2.1 Importance of *Rhopalosiphum padi* and *Metopolophium dirhodum*

Aphids occur throughout the world (Blackman and Eastop, 2007). Different cereal aphid species occur on wheat fields causing serious yield losses through their direct and indirect damage (Ehsan-ul-Haq, 2003; Nyaanga *et al*., 2006; Van Emden and Harrington, 2007; Lapierre and Hariri, 2008).

The rose-grain aphid, *Metopolophium dirhodum* Walker and the bird cherry-oat aphid, *Rhopalosiphum padi* Linneaus are two of the species most frequently found in Kenya and can reproduce parthenogenetically and viviparously to develop large populations in a short space of time (Blackman and Eastop 2000). They are minute pear-shaped, soft-bodied insects whose reproductive potential, salivary secretions, and ability to transmit viral diseases like *Barley yellow dwarf virus (*BYDV) make them among the most potent enemies of bread wheat.

The aphids initially feed on the lowest of the green leaves and as these leaves senesce they move up the plant to occupy higher leaves, feeding eventually on the flag leaf. Populations tend to decline approximately at the crop's milky ripe stage of grain development (growth stage 73–77 on Zadok's scale (Zadok, 1974) (Appendix 1). Aphids suck sap from plants and cause the leaves to appear curled and distorted, especially when the population is high. They excrete honeydew, a sugar-rich substrate that promotes the growth of sooty mold (*Capnodium* spp.) on harvestable plant parts and leaves, lowering their quality (Nyoike, 2007).

Boosting wheat production in Kenya requires improved crop varieties and effective pest control measures (Kamau, 2013). Although early infestation usually leads to severe damage (Van Emden and Harrington, 2007), aphids cause the most damage by transmitting a number of viruses, especially BYDV, for which they are the most important vectors (Riedell *et al*.,

2003; Jiménez-Martínez *et al*., 2004; Fabre *et al*., 2006; Borer *et al*., 2009 and A. Wangai, Personal communication).

2.2 Biology of *M. dirhodum* and *R. padi*

Apterous *M. dirhodum* adults have ovoid light green to yellow bodies with a length of about 2.2-2.8 mm and double dark green oblong stripe on notum. Antenna is 6-segmented, light, and slightly shorter than body length. The insect has light cylindrical siphunculi (1/6 the body length) with a small widening near base. Conic light green tail is about 1/2 length of siphunculi. Legs are light but tibial apices and tarsi are dark. Apterous *R. padi* adults are about 2 mm long) egg-shaped, pale green to black with a characteristic rusty brown tail-end, dark mid-dorsal longitudinal stripe, rather long slightly conical siphunculi with apical striae (Blackman and Eastop 2000).

Both aphid species display a diverse range of relatively complicated life cycles. Each life cycle is divided into a number of stages, with each stage characterized by one or more specialist morphs. Each of these morphs has a specific function that is necessary for the completion of each stage of the life cycle. Typical aphid life cycles have morphs that specialize in reproduction, dispersal, and surviving severe or less favourable climatic or nutritional conditions. (Helmut and Richard, 2007). In the spring, female nymphs hatch from the eggs on the primary host and feed to maturity. There are several variations of aphid life cycle. Aphids alternate primary hosts with secondary hosts, sexual with non sexual (parthenogenetic) reproduction, migrant with nonmigrant forms and wingless with winged forms. When a host becomes overcrowded, winged morphs ("alatae") develop and then spread to other plants. They may be carried over large distances by wind. Females reproduce parthenogenetically giving rise to live nymphs. Males and females are produced in the fall that migrate onto winter host where they mate to produce eggs. In the tropics and subtropics, female nymphs are produced all year round. However, in regions with mild, temperate climates, they can be anholocyclic, i.e. without a sexual generation and with aphids overwintering on the secondary host as well. Females that produce or larviposit live young are called viviparae. Hatching of fundatrix larvae is observed at daily temperatures 12-14°C

and at lower developmental threshold 5-6°C. The most favorable conditions for apterous female are the temperature 21-25°C and relative humidity 70-80%. In autumn viginoparae appear at the temperature 14-16°C and short light day. The pest gives 10-14 generations during a year (Cannon, 1985)

These aphids show very complex and rapidly changing within-year dynamics, with each clone going through several generations during the vegetative season and being made up of many individuals, which can be widely scattered in space. The survival of the eggs and/or overwintering aphids determines the numbers of aphids present the following spring. They damage agricultural and horticultural crops through direct feeding, the transmission of plant viruses, and by their impact on the aesthetic value of crops (by depositing honeydew or simply by being present). This has resulted in them being subjected, historically, to intense selection by aphicides, which has led to the evolution of a variety of resistance mechanisms (Helmut and Richard, 2007)

2.3 Control Methods of Cereal Aphids

2.3.1 Chemical Control

Since the 1960s, pest management in the industrialized countries has been based around the intensive use of synthetic chemical pesticides. Alongside advances in plant varieties, mechanization, irrigation and crop nutrition, they have helped increase crop yields by nearly 70 per cent in Europe and 100 per cent in the USA (Pretty, 2008). However, the use of synthetic pesticides is becoming significantly more difficult owing to a number of interacting factors. The injudicious use of broad-spectrum pesticides can damage human health and the environment Some of the 'older' chemical compounds have caused serious health problems among agricultural workers and others because of inadequate controls during manufacture, handling and application (Millennium Ecosystem Assessment, 2005).

Excessive and injudicious prophylactic use of pesticides can result in management failure through pest resurgence, secondary pest problems or the development of heritable resistance. Worldwide, over 500 species of arthropod pests have resistance to one or more insecticides (Hajek, 2004). Pesticide products based on 'old' chemistry are being withdrawn because of

new health and safety legislation (Pesticides Safety Directorate, 2008). However, the rate at which new, safer chemicals are being made available is very low. This is caused by a fall in the discovery rate of new active molecules and the increasing costs of registration (Thackar, 2002). Further pressures on pesticide use arise from concerns expressed by consumers and pressure groups about the safety of pesticide residues in food. These concerns are voiced despite the fact that pesticides are among the most heavily regulated of all chemicals (Chandler *et al*., 2011).

Although synthetic chemical insecticides still predominate as the primary pest control tactic, there are serious reasons to consider alternatives. The risk of synthetic chemical pesticides in the environment and their residues in human tissues was first raised by Rachel Carson in her book "Silent Spring" in 1962 (Hajek, 2004). Chemical insecticides have been documented to cause approximately 220,000 human deaths per year (Pimentel, 2008).

Chemical pesticides are generally effective against a wide range of insects and other invertebrates which each have a role and place in the ecosystem. Therefore local extinction cannot occur without disrupting the balance of nature. For instance, many species such as predators and parasites are essential as population regulators. Numerous studies have described the negative impact of chemical pesticides on non-target organisms. Extensive mortality, due to massive use of chemicals has been reported among the various animals (Hajek, 2004).

Many of the chemical insecticides currently used to control aphids are listed among the most persistent organic pollutants (POPs) by the United Nations Environmental programme (UNEP). Trade names of synthetic insecticides recommended by KALRO, Njoro for chemical control of aphids in wheat include "Karate Zeon", "Thunder", "Pirimor" and "Bullock Star" (Macharia Munene, KALRO, pers. Comm).

The most serious problems occur when pests develop resistance to chemical pesticides. The development of resistance to chemical pesticides is best illustrated with insect pests (Ambethgar, 2009). Studying the literature shows that more than 600 species of plant feeding

insect pests had developed resistance to insecticides by 1996 (Sharma et al., 2001). Resistance to pesticides among insect populations occurs when multiple applications of the same insecticide are used over multiple generations of the pest, consequently, susceptible individuals are removed from the population and resistant individuals remain to reproduce generations that can no longer be controlled with that insecticide (Riley and Sparks, 2006). The most extensively used insecticide classes - organochlorines, organophosphates, carbamates, and pyrethroids have generally been the most seriously compromised by resistance. In recent years, however, there has also been a worrying increase in resistance to more novel insecticides, including ones attacking the nervous system (neonicotinoids) and developmental pathways (benzoylphenylureas) (Bass *et al*., 2014).

Although there has been no report so far of insecticide resistance, there is cause for alarm as aphicide use in Kenya has risen, but farmers report that even with their use only 60% of crop yields are realized. The observed reduced performance of these chemicals warrants determination of whether resistance is the cause of the problem.

2.3.2 Biological Control

The use of microorganisms for biological control is commonly referred to as microbial control, an approach that includes four strategies: classical, inoculation, inundation, and conservation (Eilenberg *et al.*, 2001). Introductions of fungal entomopathogens and microsporidia for classical biological control outnumber those of other entomopathogens. Among 136 programs using different groups of arthropod pathogens, 49% have introduced fungal pathogens (Hajek and Delalibera, 2010). Recent advances in fungal production, stabilization, formulation, and application have led the way toward commercialization of a large number of new fungus-based biopesticide products (Wraight *et al*., 2001; De Faria and Wraight, 2007).

The primary advantage in the use of microbial control agents is the reduced potential for resistance development in the target pest. Resistance to *M. anisopliae* in termites has, however, has been demonstrated in the laboratory (Rosengaus *et al*., 1998; Traniello *et al*., 2002). Resistance to certain entomopathogens has also been reported under field conditions

(Asser-Kaiser *et al.*, 2007). Microbial control agents are generally considered to have little or no effect on other non-targets including beneficial insects. The relative safety to non-targets also leads to a reduced chance of secondary pest outbreaks through conservation of natural enemies (Lacey and Shapiro-Ilan, 2008). Even so, exceptions, in which entomopathogens have a negative impact on insect biocontrol agents, have been documented, e.g., a negative association between the microsporidium *N. pyrausta* and the parasitoid *Macrocentrus grandii*. In another example, entomopathogenic nematodes were observed to infect several hymenopteran parasitoids (Shannag and Capinera, 2000, Lacey et al., 2003 and Mbata and Shapiro-Ilan, 2010) or coleopteran predators (Shapiro-Ilan and Cottrell, 2005).

A primary concern in pest management is protection of human health. All additives in the microbial manufacturing product and in end-use formulations are of low toxicity and suggest little potential for human health or environmental hazard (OECD, 2003). In general, microbial control agents are safe to humans and other vertebrates, yet some exceptions exist. The bacterium *Serratia marcescens* can be an opportunistic pathogen in humans causing septicemia, formulations of *B. bassiana* can cause allergic reactions in humans (Westwood *et al.*, 2006), and in rare cases *Beauveria* sp. has been reported to be capable of causing infection in immunosuppressed humans (Henke *et al.*, 2002).

Entomopathogenic fungi produce an array of metabolites many of which are toxic or carcinogenic to vertebrates (Strasser *et al*., 2000 and Vey *et al*., 2001). However, any hazard to vertebrates would require exposure to significant levels of these metabolites. Current evidence suggests that, unless infected insects are actively pursued and ingested (insect cadavers infected with *B. bassiana* are widely consumed in China for medicinal purposes), the risk to vertebrates should be minimal. To date, no detrimental effects have been noted in vertebrates fed insect cadavers. For instance, ring-necked pheasant chicks given feed coated with *M. anisopliae* var. *acridum* (as *M. flavoviride*) spores or infected grasshoppers for 5 days showed no significant changes in weight, growth rate, behavior, or mortality rate as compared to controls fed noninoculated food or noninfected grasshoppers (Strasser *et al*., 2000). Therefore, entomopathogenic fungi should pose no obvious risk to humans because

toxin quantities produced *in vivo* are usually far lower than those produced *in vitro* and, therefore, toxin levels should never rise to harmful levels in the environment.

The application of biological control with other methods will achieve crop protection with much less harm to the ecosystem. In many parts of the world it is recognized that there has been too great a reliance on pesticide use and not enough on improving agricultural practices. There is increasing pressure to move toward minimizing pesticide usage in order to both improve the environment and to reduce cost. This is being done by using newer, more specific pesticides and by adopting improved agricultural practices and IPM, a combination of biological and chemical control (Hajek, 2004). Governments are likely to continue imposing strict safety criteria on conventional chemical pesticides, and this will result in fewer products on the market. This will create a real opportunity for biopesticide companies to help fill the gap (Herniou et al., 2003, Wang and Leger, 2005 and Muthumeenakshi, 2007).

2.3.2.1 Distribution and Abundance of Fungal Entomopathogens

Fungal entomopathogens are globally distributed in almost all terrestrial ecosystems. Diversity is at its highest in the tropical forests, but fungal entomopathogens are also found in extreme habitats such as in the high Arctic tundra (Eilenberg, 2002) and Antarctica (Bridge and Worland, 2004; Tosi *et al*., 2004). In general, the trend of global distribution patterns indicates that fungal entomopathogens in the Entomophthorales mostly occur in temperate climates, decreasing in abundance towards the subtropics and tropics although *Neozygites tanajoae* is a significant mite pathogenic fungus in tropical regions (Hajek *et al*., 2007).

The sexual stages of the Hypocreales are mostly found in tropical climates while the asexual stages are found in both tropical and temperate climates. Bidochka and Small (2005) hypothesized that anamorphic lineages of *Metarhizium* had dispersed from Southeast Asia, where the teleomorphs (*Metacordyceps*) presumably are found. From this center, most anamorphs have become cosmopolitan, although the highest *Metarhizium* diversity remains in the Asian region. It has further been hypothesized that the anamorphs such as *Metarhizium* (and presumably other genera of fungal entomopathogens in the Hypocreales) colonized managed ecosystems such as farmland around the world as generalist entomopathogens

reproducing asexually (Bidochka and Small, 2005). However, teleomorphs do occur in colder climates, although they are often restricted to pristine and mature ecosystems. Examples include, *Ophiocordyceps sinensis* in the Tibetan plateau (Weckerle *et al.*, 2010) and several *Cordyceps* species in North America (Sung and Spatafora, 2004).

The anamorphic stages of many ascomycetous fungal entomopathogens occur widely in most terrestrial ecosystems. Yet, their fungal structures are subtle in appearance and their often minute, mycosed arthropod hosts are inconspicuously located. Besides cadavers, microscopic fungal spores can be located outside their hosts in several ecosystem compartments. These compartments include predominantly the soil environment, which generally provides a stable habitat for fungal populations by buffering population fluctuations and protecting against detrimental abiotic conditions (van der Putten *et al.*, 2001).

2.3.2.2 Use of Fungal Entomopathogens as Biological Control Agents

Hajek *et al.* (2005) compiled a catalogue of pathogens and nematodes that had been released in classical biological control programs against insects and mites. The catalogue was summarized in Hajek *et al.* (2007a) and further updated with specific information on fungal pathogens (Hajek and Delalibera, 2010). Out of 136 classical biological control programs involving arthropod pathogens, 59 (43%) dealt with 20 different species of fungal entomopathogens and of these, 19 (32%) resulted in establishment. *Metarhizium anisopliae* was the most commonly introduced fungal entomopathogen (13 introductions), followed by *E. maimaiga* (seven introductions). One problem with introduction studies is that sometimes they are not conducted for long periods after the introduction, and establishment cannot be assessed. For example, Hajek *et al.* (2007a) considered this to be the case for introductions occurring after 1999.

De Faria and Wraight (2007) compiled a comprehensive list of mycoinsecticides and mycoacaricides that have been used throughout the world since the 1960s. In total, 171 products were identified, based on 12 species, mostly *B. bassiana* (34%), *M. anisopliae* (34%), *I. fumosorosea* (6%), and *B. brongniartii* (4%). Of the 171 products, 26 were no longer in use at the time of publication. The regional distribution of these products was as

follows: South America 42.7%, North America 20.5%, Europe and Asia 12.3% each, Central America 7%, Africa 2.9%, and Oceania 2.3%. Only one of the products listed is based on an Entomophthorales (*Conidiobolus thromboides*). The availability of registered products and regulatory issues involving the use of microbial pesticides in Kenya, China, India, South Korea, the European Union, Ukraine, Russia, Moldova, Argentina, Brazil, Cuba, Canada, the USA, Australia, and New Zealand are discussed by Kabaluk *et al.* (2010) and Ravensberg (2011).

There are many commercial products available worldwide, most based on fungi from six to seven species from Hyphomycetes (Copping, 2001; Alves *et al*., 2003). Over a dozen biopesticides are registered in Kenya by the Pesticide Control Products Board for control of various categories of sucking and biting insect pests. Hyphomycetes are very well suited for commercialization due to their relatively wider host ranges, ease of production, shelf life, persistence, and ease of application.

2.3.2.3 The Entomopathogenic Deuteromycetes *Beauveria bassiana* and *Metarhizium anisopliae*

For more than 120 years, *B. bassiana* and *M. anisopliae*, in addition to *B. brongniartii,* have been used to control insect pest (Zimmermann, 2007a, b). They are anamorphic fungi (asexual reproduction) distributed widely and have a wide range of host insect species (Roy *et al*., 2006; Scholte *et al.,* 2003a, 2003b). These two fungi have been developed as commercial biopesticides, for example Mycotrol® O, is produced by Mycotech using the virulent isolate of *Beauveria bassiana* strain GHA and Green Muscle® developed by the LUBILOSA project, is based on *Metarhizium anisopliae* var. *acridum* strain IMI 330189.

Both *Beauveria bassiana* and *M. anisopliae* are fungi with asexual reproduction (anamorphs) and have no sexual stage. These fungi were formerly placed in the class Hyphomycetes of the phylum Deuteromycota (also known as fungi imperfecti). The Deuteromycota is a temporary classification for fungi with no known sexual, or teleomorph stage. In this system, all fungi that have no sexual stage were placed in Deuteromycota. However, successive studies based on morphological and molecular attributes have demonstrated that some of these "imperfect

fungi" are anamorphs (asexual forms) which belong to the Phylum Ascomycota (Order: Hypocreales: Family: Clavicipitaceae) (Roy and Cottrell, 2008). It is now possible to place these fungi in their real position (Driver *et al*., 2000). *Cordyceps bassiana* is the telemorph (the sexual form) of *B.bassiana* and *Metacordyceps taii* is the telemorph of *M. anisopliae* var. *anisopliae.* Nowadays these fungi are placed in the phylum Ascomycota, class Sordariomycetes, order Hypocreales, family Clavicipitaceae and genus *Beauveria* and *Metarhizium*, respectively.

2.3.2.3.1 History, General characteristics and Taxonomy of Genus Beauveria

Beauveria is a cosmopolitan genus of soil borne EP moulds, first discovered as a devastating disease which affected European silkworms in the 18th and 19th centuries, known as the white muscardine disease (Rehner, 2005). Although the initial investigation of *Beauveria* was instigated from a need to protect domesticated insects such as silkworms and honey bees, it is also an important natural pathogen of insects, capable of infecting more than 700 species of arthropods (Lord, 2005). Its hosts include many economically important pests and its wide variation in virulence towards different insect hosts makes it one of the more versatile candidates of EP fungi for the biological control of insect pests (Rehner, 2005).

Other aspects which have contributed to the large amount of research focused on this fungal genus are firstly, its broad distribution in nature which makes *Beauveria* easy to recognize and has lead to it being noted as the most frequently encountered of all EP fungi (Rehner, 2005). Secondly, *Beauveria* is an extremely traceable organism as it can be easily isolated from insect cadavers or from the soil using simple media, antibiotics and selective agents (Rehner, 2005). Thirdly, the genus can be easily cultured in the laboratory on simple media and can be conserved by storing conidia in glycerol solutions at ultra-low temperatures or by freeze-drying (Rehner 2005). Furthermore, *Beauveria* is amenable to large-scale production and formulation as a mycoinsecticide. Despite two centuries of research and several comprehensive taxonomic studies, there are still significant problems in the identification, taxonomy and nomenclature of species in this genus and progress in elucidating the genetics and evolution of *Beauveria* is just beginning (Rehner, 2005; Rehner & Buckley 2005).

Vuillemin (1912) formally described the genus *Beauveria*, assigning *Botrytis bassiana* as the type species, this later became *Beauveria bassiana* in recognition of J. Beauverie in 1914, after his invaluable contribution to the study of white muscardine disease (Rehner, 2005). The genus is easy to distinguish morphologically. It's most distinctive features are the sympodial to whorled clusters of short-globose to flask-shaped conidiogenous cells that produce a succession of one-celled, sessile and hyaline conidia on an elongating „zigzag" rachis (Domsch *et al.*, 2007) (Fig. 2.1 A). In culture, *Beauveria* species typically produce white coloured mycelium and conidia (Fig. 2.1 B), although some isolates may produce yellow pigment in the older, central parts of the colony (Domsch *et al.*, 2007). Colony growth tends to be rapid, and the texture of the mycelium is typically lanate to woolly and synnemalike projections can occasionally be observed (Rehner, 2005). *Beauveria* conidial production can often be profuse, frequently creating a chalky, mealy or powdery appearance on the colony surface (Rehner, 2005). Many isolates can excrete a red pigment into the culture medium, although this does not occur under all culture conditions (Rehner, 2005).

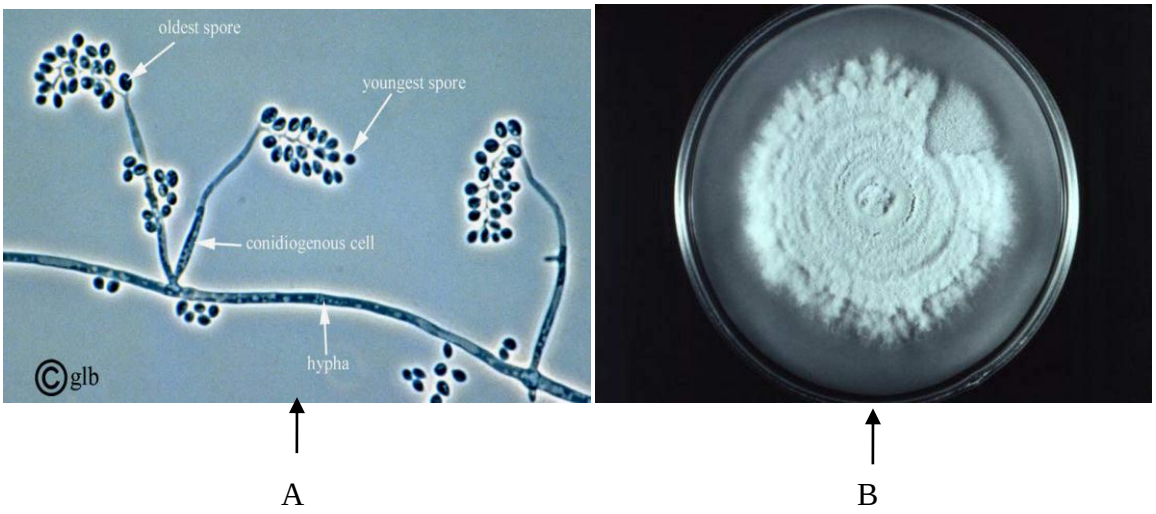

Figure 2.1 (A) A photomicrograph showing three conidiogenous cells on a hypha of *Beauveria bassiana*. The asexual conidia are produced in acropetal succession (youngest at the tip) (B) *B. bassiana* grown on Sauboraud dextrose agar (SDA) at 25 °C. Photo Credits: (A) George Barron, University of Guelph, Canada (B) T. Goble, Rhodes University, South Africa.

The conidial form is the only morphological feature of the genus that has proven helpful for species delineation, as conidia vary from globose, ellipsoid, cylindrical and vermiform and range in sizes from 1.8 to 6.0 μm in their greatest dimensions (Rehner, 2005; Domsch *et al.*, 2007). In total 49 species have been classified in the genus of which 22 are currently considered valued (Rehner, 2005). Overlapping variation in the size of conidia among many species has raised questions of the validity of routine species identifications. Further, cultural features of *Beauveria* are influenced by culture conditions and are thus unreliable as taxonomic features (Rehner, 2005). Six morphological species can be discerned in the genus *Beauveria*: *B. amorpha*, *B. bassiana*, *B. brongniartii*, *B. caledonica*, *B. velata* and *B. vermiconia* (Fig. 2.2). There is however much confusion with regard to the species *B. bassiana* for which many morphologically similar species have been described; as it stands seven species are synonymous with *B. bassiana* (Rehner, 2005).

Current molecular techniques based on the comparison of available internal transcribed spacer (ITS) sequences and other molecular markers among species, has shown great promise in elucidating the genetic taxonomy of this genus (Rehner and Buckley, 2005; Meyling *et al.*, 2009). An important inference obtained using molecular analyses was the discovery of the close relatedness between the asexual or anamorphic genus *Beauveria,* and the sexual or teleomorphic genus *Cordyceps. Beauveria bassiana s.s.* reproduces asexually by mitosporic conidia and for some time it has been presumed to be exclusively clonal (Meyling *et al.*, 2009). Phylogenetic and developmental studies have linked *B. bassiana s.s.* to the Asian sexual species *Cordyceps bassiana* which provides evidence that *B. bassiana* is in fact facultatively sexual (Meyling *et al.*, 2009).

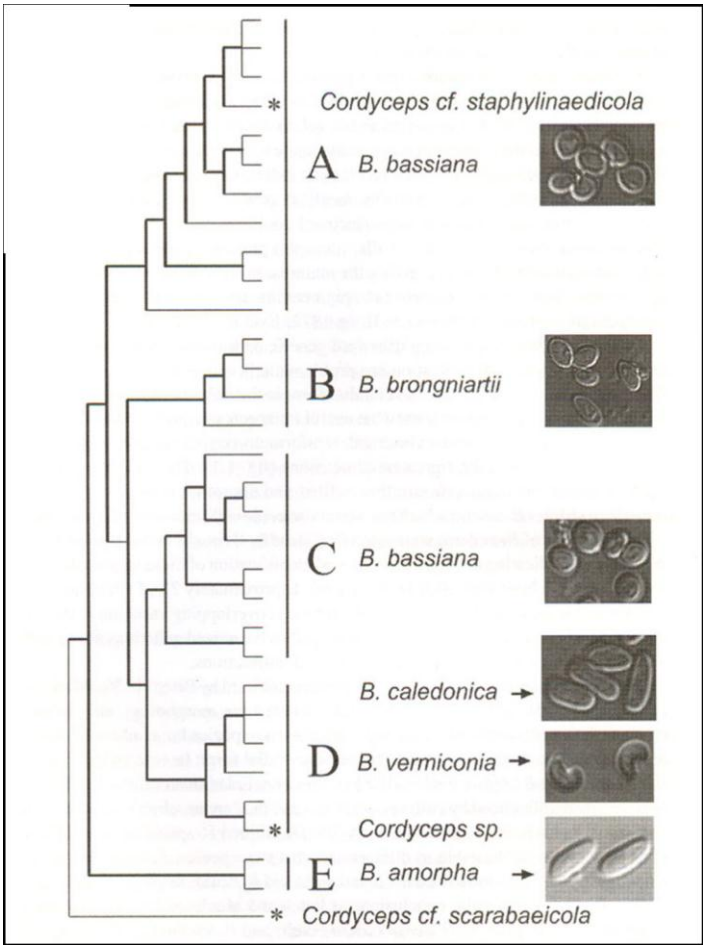

Figure 2.2 The phylogenetic relationships of the genus *Beauveria* based on several independent Bayesian and parsimony analyses. Clades A-E are labelled according to their morphological species identification and micrographs of representative conidia (Rehner, 2005).

2.3.2.3.2 History, General Characteristics and Taxonomy of Metarhizium

Metarhizium is a haploid cosmopolitan genus of soil borne EP moulds. Elie Metchnikoff, a Russian microbiologist, was the first person to isolate and name the green muscardine fungus, *Entomophthora anisopliae* in the 1880s (now known as *Metarhizium anisopliae*) which was found infecting the grain beetles he worked on (Lord, 2005). *Metarhizium* species have a wide range of virulence and are known to infect more than 200 different insect species, many of which are major agricultural pests, such as sugarcane stem-borers, scarab grubs and grasshoppers (Bidochka and Small, 2005). Further *Metarhizium* is capable of

infecting insects from over 14 different orders including some non target orders such as Malacostrata (Amphipods), Acari, Ephemeroptera and Dermaptera (Zimmermann, 2007a).

As with *Beauveria*, the earlier classification of this genus was based on morphological characteristics, but because these characteristics are fairly limited it has complicated the taxonomy (Bidochka and Small, 2005). The genus is morphologically defined on the basis of the arrangement of the phialides which bear chains and columns of green, dry and slightly ovoid conidia (Zimmermann, 2007b) (Fig. 2.2 A,B). Tulloch used the morphological characteristics of the conidia to distinguish between the two forms of *M. anisopliae;* the short-spore (5-8 μm) form called *M. anisopliae* var. *anisopliae* and the long-conidial form (10-16 μm) called *M. anisopliae* var. *majus* (Zimmermann, 2007b). Since then nine additional taxa have been described, although each may not be formally recognized as a separate *Metarhizium* species (Bidochka and Small, 2005).

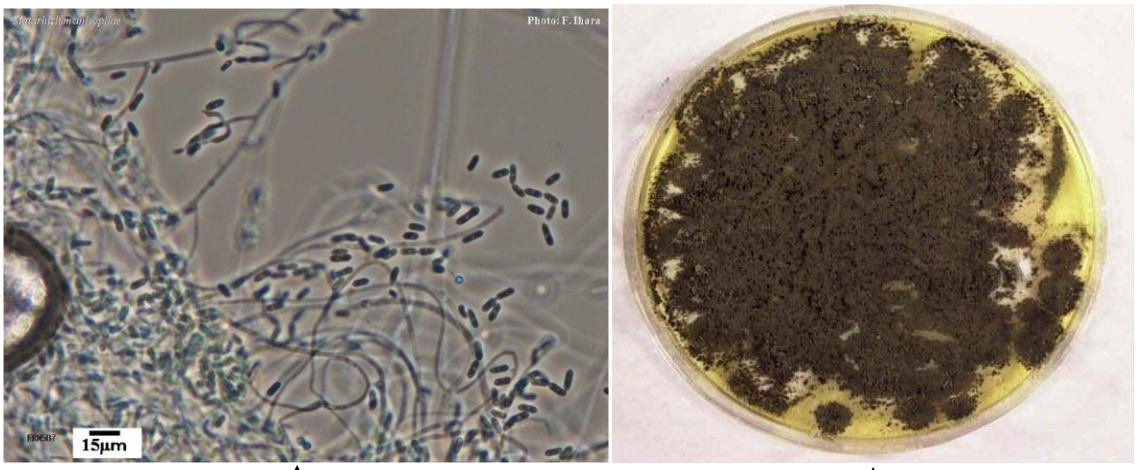

Figure 2.3 (A) A transmission electron micrograph showing conidiogenous cells on the hyphae of *Metarhizium anisopliae* (B) *M. anisopliae* grown on Sauboraud dextrose agar (SDA) at 25 °C. Photo Credits: (A) Fumio Ihara, National Institute of Fruit Tree Science, Japan (B) T. Goble, Rhodes University, South Africa.

Several commercial endeavours have registered strains of *Metarhizium* as the active ingredient of various products for insect pest management namely: *Metarhizium* 50®

(AgoBiocontrol) for garden pest control in Columbia; Biogreen® and Green Guard® in Australia for red-headed cockchafer, *Adoryphouse couloni* and locusts respectively, BIO 1020® in Germany for vine weevils, *Otiorhynchus sulcatus* and Green Muscle® (Biological Control Products, South Africa) against locusts, *Locustana pardalina* and grasshoppers (Bidochka and Small, 2005). Many other registered *Metarhizium* products are currently available and a comprehensive list can be found in De Faria and Wraight (2007). There are many criteria which become important in selecting a strain of *Metarhizium* for commercialisation: a high level of virulence towards the target pest, the utilisation of indigenous strains is preferable because exotic strains could be politically and ecologically problematic. Finally, the ability to distinguish and track the formulated strain against background populations of EP fungi, either through genotyping or genetic tagging becomes crucial to ascertaining the effectiveness of the applied strain (Bidochka and Small, 2005).

2.3.2.4 Fungal Infection Process and Insect Responses to Infection

All EP fungi have the same basic mode of action when infecting insect hosts and are unique in that they can infect insect hosts through the cuticle (Goettel 1995). The conidia, or asexual spores, come into direct contact with the host and adhere to the cuticle via nonspecific hydrophobic mechanisms (Inglis *et al.,* 2001). Under specific environmental conditions the conidia germinate and a germ tube, or appressorium (penetration-peg structure), are produced (Inglis *et al.,* 2001) (Fig. 2.4). The cuticle is penetrated by a combination of mechanical pressure from the appressorium and the action of cuticle-degrading enzymes, such as trypsin, metalloproteases and aminopeptidases (Bidochka and Small, 2005). The fungus grows by vegetative growth in the host haemocoel and external conidia are produced upon death of the host when fungal hyphae exit through the less sclerotised areas of the cuticle (Inglis *et al.,* 2001) (Fig. 2.4).

Some EP fungal species, such as *Metarhizium* and *Beauveria,* produce powerful insecticidal cyclic peptides called destruxins and a toxic metabolite called oosporein (Inglis *et al.* 2001). Comprehensive reviews on the function and mode of action of destruxins and oosporeins are reported in the literature (Zimmermann, 2007a, b). Fungal strains which produce these toxins are sought after for commercialisation because although fungi grow sparsely in the insect

haemocoel, they kill the insect relatively quickly. Strains which do not produce these toxins grow profusely in the insect haemocoel but take much longer to kill their hosts (Bidochka and Small, 2005). The host cuticle is the first line of defense against fungal infection and has a central role in determining fungal specificity. If the fungus breaches the cuticle, successful infection can only occur if the fungus can overcome the innate immune response of the insect

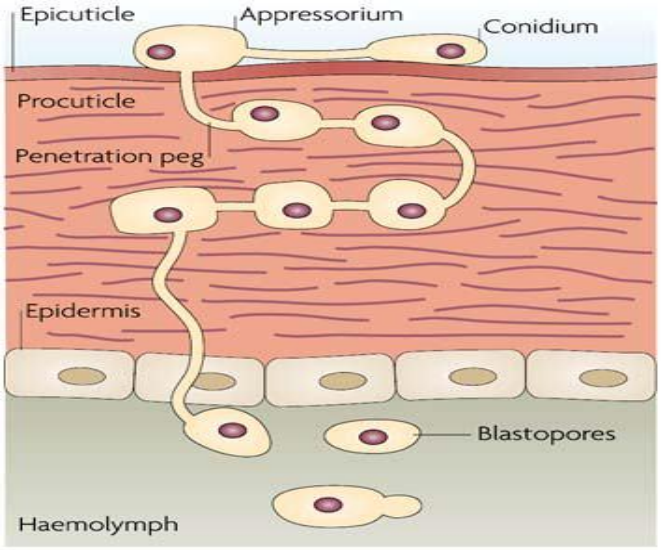

Figure 2.4 Schematic diagram of an EP fungus invading the host insect cuticle. Photo Credits: Thomas and Read 2007, University of Texas Austin.

Insect behavior can alter the success of fungal entomopathogen infection. In theory, social insects are at greater risk of infection by pathogens because many individuals live close together and are often closely related. However, social insects have evolved a range of mechanisms to defend against pathogens (Cremer *et al.*, 2007). For example, termites (Yanagawa *et al*., 2008) exhibit mutual grooming behavior to remove conidia from themselves and from colony members. Being social insects, termites have developed a specific set of responses to guide healthy members to ensure that infection is not transmitted to the rest of the colony (Mburu *et al*., 2009). Furthermore, the metapleural glands of ants produce antibiotic secretions that inactivate fungal entomopathogens (Hughes *et al*., 2002; Yek and Mueller, 2011). Cremer *et al.* (2007) list a range of mechanisms by which social insects can combat pathogen establishment. Nielsen *et al.* (2010) showed that aphid-tending

ants remove sporulating aphid corpses infected with *P. neoaphidis* and will also groom aphids and remove conidia from the aphid cuticle, possibly to protect their mutualistic honeydew-producing resource.

Some non-social insects have been shown to exhibit density-dependent prophylaxis, i.e., increased resistance to pathogens when reared under high-density conditions (Wilson *et al.*, 2001; Barnes and Siva-Jothy, 2010). This increased resistance appears to be related to cuticular melanization, the formation of the pigment melanin by polymerization of phenolic compounds (Jacobson, 2000). In contrast, the increased resistance against *M. acridum* in *S. gregaria* reared in crowded conditions appears to be related to increased antimicrobial activity (Wilson *et al.*, 2002).

Once the fungal entomopathogen reaches the hemolymph, a range of immune responses can be initiated. Some are general antimicrobial responses while some are specifically targeting the invading fungus (Rolff and Reynolds, 2009). *Metarhizium anisopliae* and *B. bassiana* are capable of avoiding encapsulation in the hemocoel, and this adaptation has been hypothesized to be a consequence of these fungi being facultative entomopathogens in soil environments where they can survive encapsulation by soil amoeboid predators (Bidochka *et al.*, 2010). A fascinating response to fungal infections is the concept of behavioral fever (Roy *et al.*, 2006), whereby infected insects (e.g., locusts, flies) modify their behavior (e.g., basking in the sun) to increase body temperature, with an adverse effect on the fungal entomopathogen present in the hemocoel (Kalsbeek *et al.*, 2001; Elliot *et al.*, 2002).

The time from when the insect enters and infects the host until the insect dies is referred to as the period of lethal infection (Figure 2.5), or if the insect recovers as the period of infection. A short period of lethal infection indicates that the entomopathogen has high virulence, whereas a long period of lethal infection indicates that it has low virulence. An entomopathogen with high virulence is not necessarily the most infectious. For example, *Bt* subspecies *kurstaki* has high virulence to cabbage butterfly (*Pieris rapae*) larvae with a period of lethal infection of 48 h. But high virulence does not mean that the entomopathogen is highly contagious, and *Bacillus thuringiensis* subspecies *kurstaki* is not easily transmitted

from one insect to another. Conversely, an entomopathogen with low virulence (i.e., period of lethal infection of several weeks as occurs with some microsporidian infections) may be highly contagious, with the pathogen being easily transmitted from one insect to another

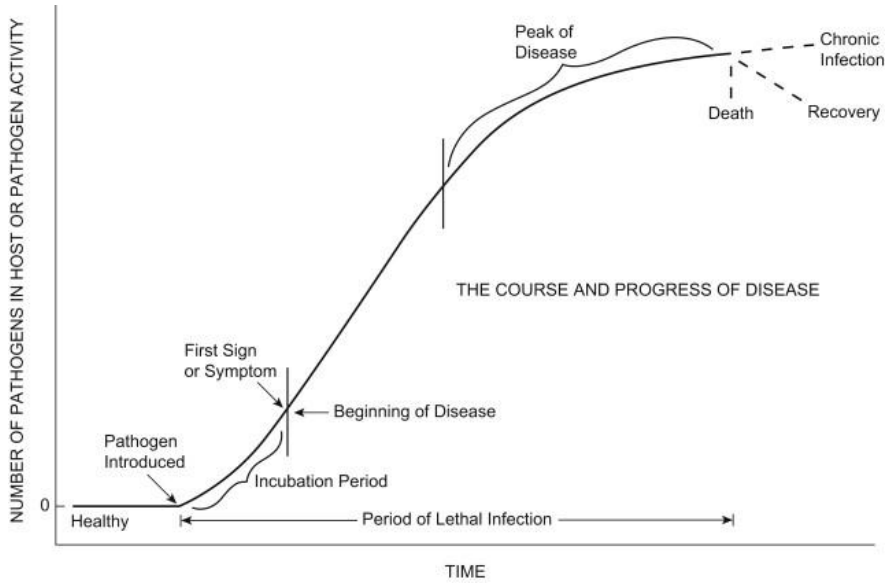

Figure 2.5 Sequence of events constituting the course and progress of disease following acquisition of infectious concentration of an entomopathogen by a healthy susceptible insect. (Modified after Tanada and Kaya, 1993)**.**

2.3.2.5 Factors Affecting Efficacy in Microbial Control

Beyond safety and possessing a low potential for resistance, some other factors affect the efficacy of microbial control agents. An ideal microbial control agent is likely to possess the following attributes: (1) a high level of virulence to the target pest; (2) ease of production and storage; and (3) the ability to persist in the environment.

Virulence has a direct effect on microbial control efficacy. Pathogens, even at the strain level, can vary greatly in their ability to cause high levels of mortality in a specific host. Speed of kill or disease onset can also vary greatly among pathogens. Some pathogens do not cause mortality directly; for example, although there are a few exceptions (e.g., *Vairimorpha necatrix*, which can rapidly cause mortality), most microsporidia cause chronic infections

that may affect various aspects of host fitness such as reproductive capacity, longevity, or development without causing direct mortality (Solter and Becnel, 2007). Fast-acting pathogens can be disadvantageous when targeting social insects, which may impair virulent pathogens through avoidance and removal of infected individuals (Rath, 2000; Wilson-Rich *et al*., 2007 and Wilson-Rich *et al*., 2009). In contrast, chronic pathogens can have the advantage of infiltrating social insect colonies, including accessing the queen (Oi, 2006; Milks *et al*., 2008).

The pathogen population density affects microbial control efficacy. A higher pathogen population density (as well as a higher host density) leads to increased host–pathogen contact. Thus, in susceptible hosts, it is assumed that a certain pathogen density is required to suppress the target pest below an economic injury level (EIL), and that lower densities will fail. There are many examples that demonstrate this dose–response relationship between pathogen and host in the laboratory, using fungi (Hesketh *et al*., 2008; Wraight *et al*., 2010), nematodes (Power *et al.*, 2009), or viruses (Figueiredo *et al.*, 2009). Effects of pathogen application rate in field studies have been reported (Lacey and Kaya, 2007; Lacey and Shapiro-Ilan, 2008), using nematodes (McCoy *et al*., 2002, Arthurs *et al*., 2005; Chambers *et al*., 2010) or fungi (Wraight and Ramos, 2002), whereas in other studies the effects of field application rates varied, were not detected, or were not deemed important relevant to other factors (strain or species effect) (Cappaert and Koppenhöfer, 2003, Grewal *et al*., 2004; Dillon *et al*., 2007).

In addition to susceptibility to UV radiation, entomopathogenic nematodes are highly sensitive to desiccation (Shapiro-Ilan *et al*., 2006) and fungal entomopathogens generally require high levels of relative humidity for germination. Yet, exceptions exist for the fungal requirement of high humidity (Wraight *et al.*, 2007); e.g., low humidity was reported to be beneficial for control of the lesser grain borer, *Rhyzopertha dominica* with *B. bassiana* (Lord, 2005). Furthermore, high levels of moisture in the soil can be detrimental to fungi by enhancing environmental degradation through increased antagonists in soil (Shapiro-Ilan *et al*., 2004a, Jaronski, 2007; Wraight *et al*., 2007). A number of other factors can affect entomopathogen persistence in soil, including pH, texture, aeration, antagonists, temperature,

and use of amendments (fertilizers or pesticides) (Lacey *et al*., 2001, Shapiro-Ilan *et al*., 2006; Meyling and Eilenberg, 2007).

Achieving or improving efficacy in microbial control can rely on choosing the best entomopathogen for a particular system. The most suitable entomopathogen from a variety of candidates can be selected simply by screening existing species and strains that possess superior desired traits such as virulence and environmental tolerance. New entomopathogens can be discovered through surveys and screened in parallel to existing strains. Such surveys have been conducted extensively for entomopathogenic nematodes (Shapiro-Ilan *et al*., 2003a, Shapiro-Ilan *et al*., 2008a) and fungi (Leland *et al*., 2005, McGuire *et al*., 2005; Lubeck *et al*., 2008). The screening process is often accomplished by first narrowing down the number of candidates in laboratory comparisons. Such comparisons have been made to find superior entomopathogen strains for numerous target pests such as the emerald ash borer, *Agrilus planipennis* (Castrillo *et al.*, 2010), aphids (Shapiro-Ilan *et al.*, 2008a), cowpea weevils, *Callosobruchus maculatus* (Cherry *et al.*, 2005), plum curculio, *Conotrachelus nenuphar* (Shapiro-Ilan *et al.*, 2008b), *Lygus* spp. (Liu *et al.*, 2002 and Leland *et al*., 2005), *O. sulcatus* (Bruck, 2004), and mites (*Tetranychus cinnabarinus*) (Shi and Feng, 2004).

The importance of verifying the results of laboratory efficacy in the field cannot be overemphasized. An entomopathogen that shows high virulence in the controlled environment of a laboratory could fail to suppress the target pest in the field owing to various biotic or abiotic factors that render the organism incompatible. A lack of understanding of the biological and ecological constraints required for pathogen persistence and proliferation in the environment is likely to lead to a discrepancy between laboratory and field efficacy (Hu and St. Leger, 2002; Bruck, 2005; Bruck, 2010). Some examples of laboratory screening studies that selected entomopathogen strains or species that later proved successful in the field include *S. riobrave* and *H. indica* for control of the citrus weevil, *Diaprepes abbreviatus*, *S. riobrave* for control of *C. nenuphar* (Shapiro-Ilan *et al*., 2002b, Shapiro-Ilan *et al*., 2004b; Pereault *et al*., 2009) and *M. anisopliae* for control of *A. ludens* (Lezama-Gutiérrez *et al.*, 2000).

In contrast, in some cases a high level of laboratory virulence or efficacy has not been corroborated under field conditions. For instance, *S. feltiae* was highly virulent to *C. nenuphar* in the laboratory, but failed to control the pest in Georgia peach orchards, possibly because of unsuitable soil temperatures (Shapiro-Ilan *et al.*, 2004b). A focus on pathogen ecology and habitat preferences when selecting strains for microbial control is expected to enhance the pathogen performance in the field and the frequency and magnitude of epizootics (Jaronski, 2007; Jaronski, 2010 and Vega *et al*., 2009).

Successful microbial control applications can be facilitated through improved formulation. Research on improved entomopathogen formulations is directed toward ease of handling and enhanced persistence in the environment. Significant work has been undertaken to mitigate the effect of UV radiation on entomopathogens via improved formulation of microbial control products (Jackson et al., 2010). Recent advances in formulation of entomopathogenic nematodes that have facilitated above-ground use, a major barrier to expanding use of this pathogen group, using mixtures with a surfactant and polymer (Schroer and Ehlers, 2005), postapplication protective covers using foam (Lacey *et al.,* 2010), and a sprayable gel thought to provide resistance to UV radiation and desiccation (Shapiro-Ilan *et al.*, 2006). UV radiation-protecting formulations have also been developed for fungal entomopathogens (Behle *et al.*, 2011), Protective formulations have also substantially improved the persistence of Bt (Garczynski and Siegel, 2007). Bait formulations can enhance entomopathogen persistence and reduce the quantity of microbial agents required per unit area; e.g., baits have been developed for entomopathogenic nematodes (Grewal, 2002) and fungi (Geden and Steinkraus, 2003)

Entomopathogen efficacy may also be enhanced through combination with physical agents. For example, a synergistic effect of diatomaceous earth combined with *B. bassiana* has been observed with a number of coleopteran stored grain pests (Lord, 2001, Akbar *et al*., 2004; Athanassiou and Steenburg, 2007) as well as with the microsporidium *T. solenopsae* infecting the red imported fire ant (Brinkman and Gardner, 2001). While the exact details of the interaction between diatomaceous earth and pathogens are currently unclear, it appears to involve a combination of increased availability of water and other nutrients, removal or

mitigation of inhibitory materials, alteration of adhesive properties, and physical disruption of the cuticular barrier (Akbar *et al.*, 2004).

CHAPTER THREE

SCREENING DIFFERENT ISOLATES OF *METARHIZIUM ANISOPLIAE* AND *BEAUVERIA BASSIANA* FOR PATHOGENICITY AGAINST *RHOPALOSIPHUM PADI* (LINNEAUS) AND *METOPOLOPHIUM DIRHODUM* (WALKER)

3.1 Introduction

Different cereal aphid species occur on wheat fields causing serious yield losses through their direct and indirect damage (Ehsan-ul-Haq, 2003; Nyaanga *et al*., 2006; Van Emden and Harrington, 2007; Lapierre and Hariri, 2008). The rose-grain aphid *Metopolophium dirhodum* (wlk.), along with the bird cherry-oat aphid, *Rhopalosiphum padi* (Linneaus), are two of the species most frequently found in Kenya. They can reproduce parthenogenetically and viviparously to develop large populations in a short space of time (Blackman and Eastop 2000).

Although these aphids can remove considerable amounts of liquid and nutrients from phloem, and strong infestations can sometimes lead to leaf distortion, the direct effect on grain yield is generally minor. However, if plants are infested when they are young, significant direct damage is done. Both aphid species cause the most damage by transmitting a number of viruses, especially *Barley yellow dwarf virus* (BYDV), for which they are the most important vector (Riedell *et al*., 2003; Jiménez-Martínez *et al*., 2004; Fabre *et al*., 2006; Borer *et al*., 2009).

A major problem facing the bread wheat industry in Kenya is that most commercial varieties are susceptible to *R. Padi* and *M. dirhodum.* Aphicide use in Kenya against these pests has risen, but farmers report that even with their use only 60% of crop yields are realized. The current methods of controlling aphids have been largely unsuccessful and involve the use of systemic insecticides both as seed dress and foliar spray. Moreover, many of the chemical insecticides currently used to control aphids are listed among the most persistent organic pollutants (POPs) by the United Nations Environmental programme (UNEP). Fungi as microbial insecticides could provide an environmentally benign alternative to the ever-

increasing pesticide use for aphid vector management. Fungal pathogens are relatively host specific with minimal effect on non-target beneficial organisms and could be compatible with other IPM programmes. They are known to have low mammalian toxicity. Studies confirm that insect pathogenic fungi are important in agricultural systems (Klingen *et al*., 2002). The two entomogenous fungi (*M. anisopliae* and *B. bassiana)* have been studied and applied to manage various insect pests on many crops around the world (Copping, 2000; Goettel *et al*., 2000; Nugroho and Ibrahim, 2007 and Yubak *et al*., 2008).

The most suitable entomopathogen from a variety of candidates can be selected simply by screening existing species and strains that possess superior desired traits such as virulence and environmental tolerance. The screening process is often accomplished by first narrowing down the number of candidates in laboratory comparisons. Such comparisons have been made to find superior entomopathogen strains for numerous target pests such as the emerald ash borer, *Agrilus planipennis* (Castrillo *et al.*, 2010), aphids (Shapiro-Ilan *et al.*, 2008a), cowpea weevils, *Callosobruchus maculatus* (Cherry *et al.*, 2005), plum curculio, *Conotrachelus nenuphar* (Shapiro-Ilan *et al.*, 2008b), *Lygus* spp. (Liu *et al*., 2002 and Leland et al., 2005), *O. sulcatus* (Bruck, 2004), and mites (e.g., *Tetranychus cinnabarinus*) (Shi and Feng, 2004).

The success of entomopathogenic fungi as microbial control agents require careful and appropriate selection of the most efficacious species and isolate. In this study, six isolates of *B. bassiana* (Bals.) Vuill. and fourteen isolates of *M. anisopliae* (Metsch.) Sorok were evaluated in the laboratory for their pathogenicity to *M. dirhodum (Walker) and R. padi* (Linneaus).

3.2 Materials and Methods

3.2.1 Study site

Laboratory experiments were carried out at the Arthropod Pathology Unit of the International Centre for Insect Physiology and Ecology (*icipe*), Duduville Campus in Nairobi, Kenya.

3.2.2 Maintenance of aphid colony/rearing.

The rearing of the aphids was carried out according to the method described by Knudsen *et al.*, (1994). The colony was maintained at ICIPE's screen house on potted caged wheat, *Triticum aestivum* L. (variety- Mbuni) seedlings. Seedlings were grown in autoclaved potting mix (sand + forest soil) in plastic pots (10 cm diameter, 15 cm deep), with a photoperiod of 12:12 (L: D) at 26 ± 3°C. Aphids were transferred to new plants on a weekly basis. To obtain similar-aged cohorts, a group of 30 adult *Metopolophium dirhodum and Rhopalosiphum padi* were transferred onto leaf sections inserted into moist cotton wool in 12 x 12 cm meshed Petri dishes and left for 48 h. The adults were then removed, leaving a cohort of new-born nymphs of similar age (1 - 2 d old). These nymphs were maintained for an additional 2 - 3 d before use in experiments.

3.2.3 Fungal isolates

Twenty isolates of *B. bassiana* and *M. anisopliae* (Table 3.1) were used in this study. These were sourced from *icipe*'s Arthropod Germplasm Centre. The fungi were grown for 21 days on Sabouraud dextrose agar (SDA) plates at 26 ± 2°C. Conidia were harvested by scraping the surface using a sterile rubber. Inocula were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads. Conidial suspensions were vortexed for 5 min to produce a homogenous suspension. Spore concentrations were determined using a haemocytometer.

3.2.4 Conidial germination tests

Germination tests were performed on all isolates of the two fungi species used in the mortality and dose-response tests. Viability tests for each bioassay were carried using the technique described by Goettel and Inglis (1997) by spread-plating 0.1 ml of conidial suspension titrated at 3.0 x 10^6 conidia/ml on SDA plates. Four sterile cover slips (22 × 22 mm) were placed individually on different locations on each plate. The plates (n = 6 per replicate) were sealed with parafilm and incubated at 26 ± 2ºC and 90 ± 5% RH and examined between 15-18 hours under phase contrast microscope. The percentage germination of conidia was determined from 100 spore counts under cover slips at ×40 magnification. Only those conidia with germ tubes with the same length as the diameter of

the conidia were designated as having germinated (Figure 3.1). Over 88 % of conidia germinated in all the tests (Table 3.1).

Table 3.1: Viability of various isolates of *M. anisopliae* and *B. bassiana* screened against *R. padi* and *M. dirhodum*.

Species/Isolate/ Depositor's No.	ICIPE Accession No.	Origin of isolates, Locality, Country	% germination	Year of isolation
Metarhizium anisopliae				
ICIPE 20	ICIPE 20	Soil, Migori, Kenya	90.6 ± 0.8	1989
ICIPE 41	ICIPE 41	Lemba, DRC	88.6 ± 0.9	1990
ICIPE 60	ICIPE 60	Kabello, DRC	92.7 ± 1.2	1990
ICIPE 78	ICIPE 78	*T. nigroplagia* Ungoe, Kenya	92.9 ± 1.0	1990
ICIPE 84	ICIPE 84	Sorghum Kendu Bay, Kenya	95.0 ± 1.0	1989
ICIPE 62	ICIPE 62	Kinshasa, DRC	90.7 ± 0.9	1990
ICIPE 69	ICIPE 69	Matete, DRC	92.1 ± 1.0	1990
GM	ICIPE 23	Niger	95.8 ± 0.7	1998
SP9	ICIPE 31	Madagascar	96.0 ± 0.7	2003
GATEGI	ICIPE 98	Embu, Kenya	90.6 ± 0.6	2005
EMBU 27	ICIPE 51	Embu, Kenya	93.8 ± 0.8	2005
KITUI 13	ICIPE 25	Kitui, Kenya	91.1 ± 1.5	1999
ICIPE 30	ICIPE 30	*Busseola fusca* Kendu Bay	90.5 ± 0.9	1989
GPK/RI/MA	ICIPE 07	*A. variegatum* Rusinga, Kenya	88.6 ± 0.6	1996
Beauveria bassiana				
BBKERICHO	ICIPE 279	Kericho, Kenya	95.9 ± 0.8	2005
BBMBITA	ICIPE 273	Mbita, Kenya	90.6 ± 1.0	2004
GPK/RI/BB	ICIPE 271	Mombasa, Kenya	90.8 ± 0.9	1996
NKOMETOU	**	Mauritius	88.4 ± 0.8	2005
BBM 573	ICIPE 285	Mauritius	90.3 ± 0.8	2005
BB 236	ICIPE 294	Mauritius	90.6 ± 0.9	2005

** Accession Number not yet designated

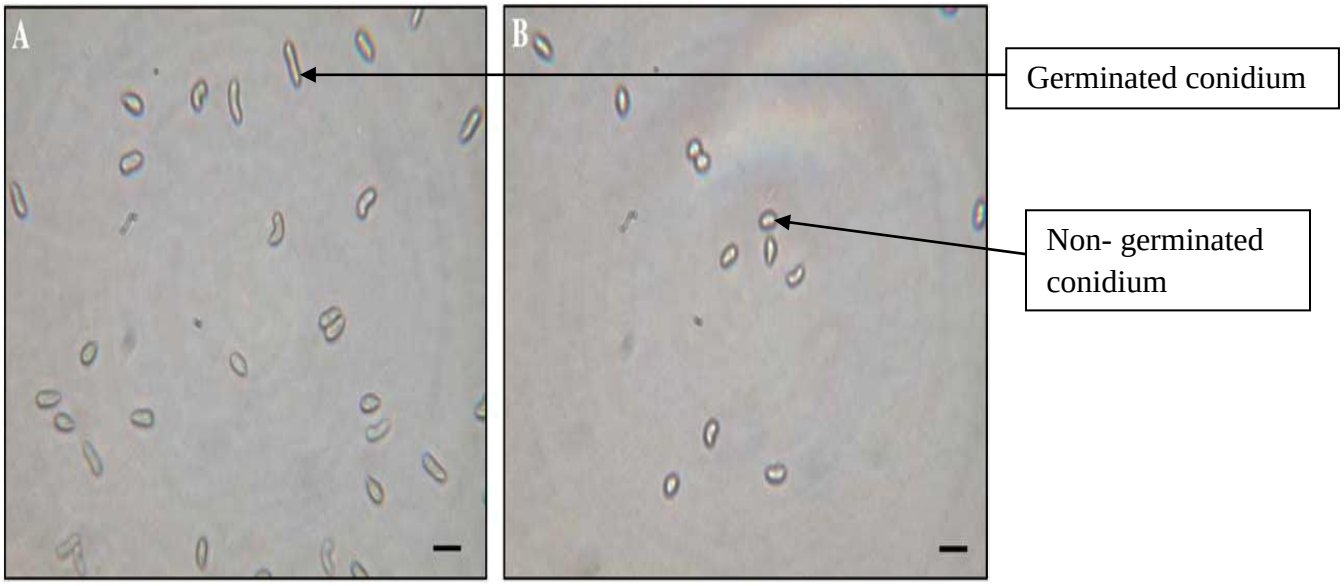

Figure 3.1 Germinated (A) and non-germinated (B) conidia of *Metarhizium anisopliae* at x 400

3.2.5 Insect inoculation

A standard concentration of 10^7 conidia ml^{-1} was used for screening. Both sides of fresh "Mbuni" wheat seedling leaves were sprayed with 10 ml conidial suspension using Burgerjon's spray tower (Burgerjon, 1956) (Figure 3.2). Control lots were treated with sterile distilled water containing 0.05% Triton X-l00. The spray tower was cleaned with 90% alcohol and sterile distilled water between treatments. Screenhouse maintained 3 – 5 day old aphids were then carefully removed by camel hair brush and placed on the treated wheat seedling leaves whose end was inserted into moist cotton wool to create a high humidity environment along one side of 12 x 12 cm Petri dishes. The top part of Petri dish lids were cut and replaced with meshed net to provide aeration (Figure 3.3). Test aphids were exposed to treated wheat leaf discs for 4 days, after which treated discs were removed and replaced with fresh and untreated leaf discs. The Petri dishes were placed on racks under normal screenhouse conditions at temperatures of 26 ± 2°C and photoperiod of 12:12 (Light/Darkness). Treatments consisted of 20 aphids each and were replicated five times.

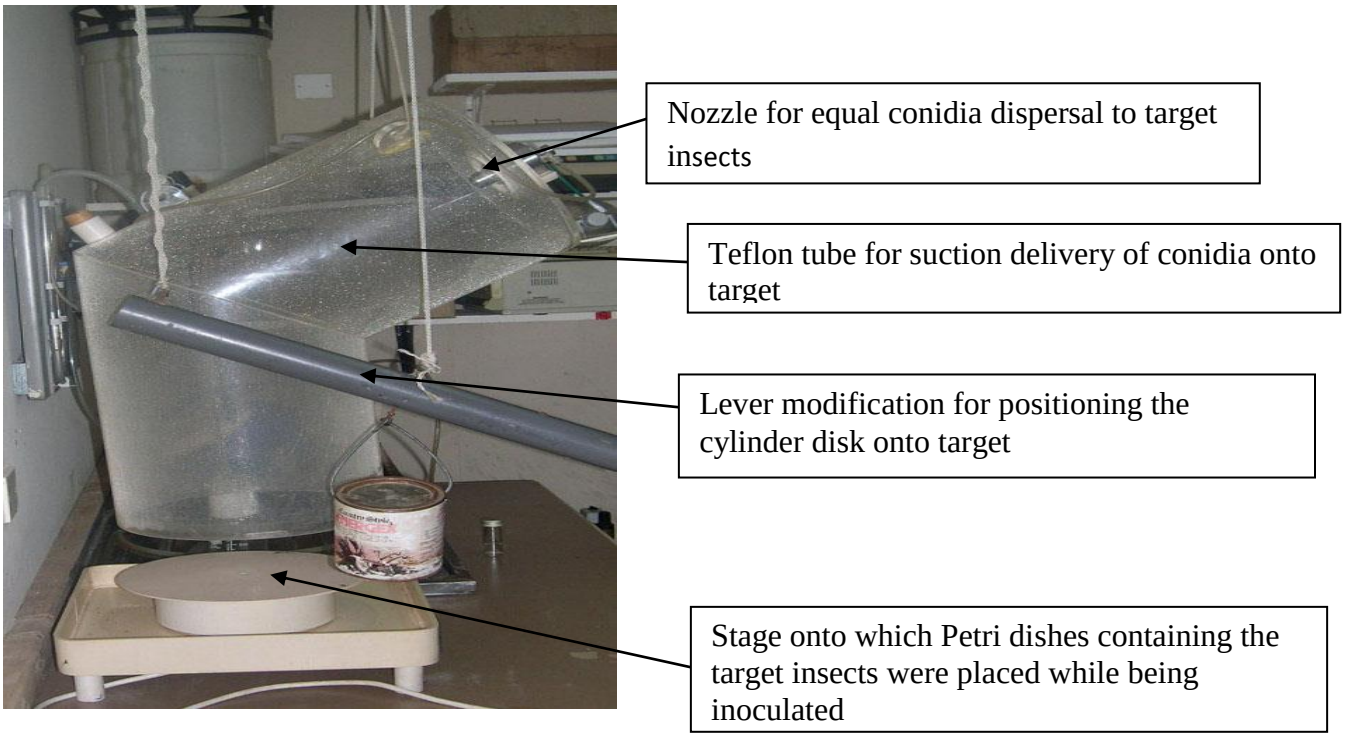

Figure 3.2 Burgerjon spray tower used to spray conidial suspension onto aphids

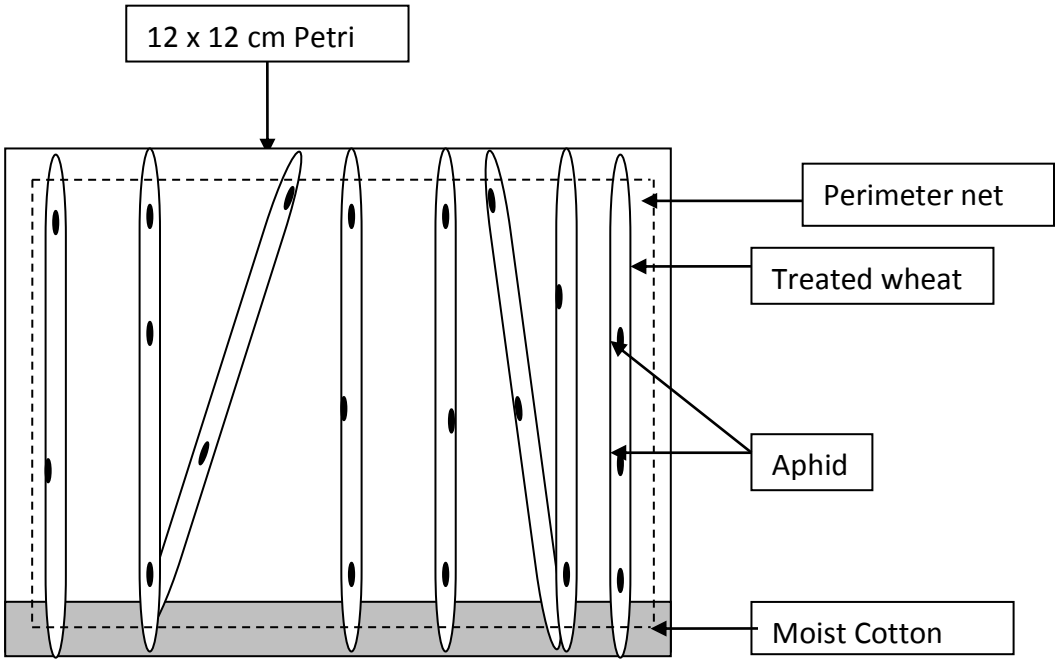

Figure 3.3 Layout of experimental unit for Screening of aphid fungal pathogens

Mortality was recorded daily for 10 days post-infection. An aphid was considered dead if it did not respond by moving when touched with the tip of a fine camel's hair brush. Dead aphids were transferred to slides in Petri dishes lined with moist filter paper to allow the growth of the fungus on the surface of the cadavers. Mycoses were confirmed by daily microscopic examination of hyphae and spores (Figure 3.4) at a magnification of x 400. Only mortality by mycosis was considered in the present report.

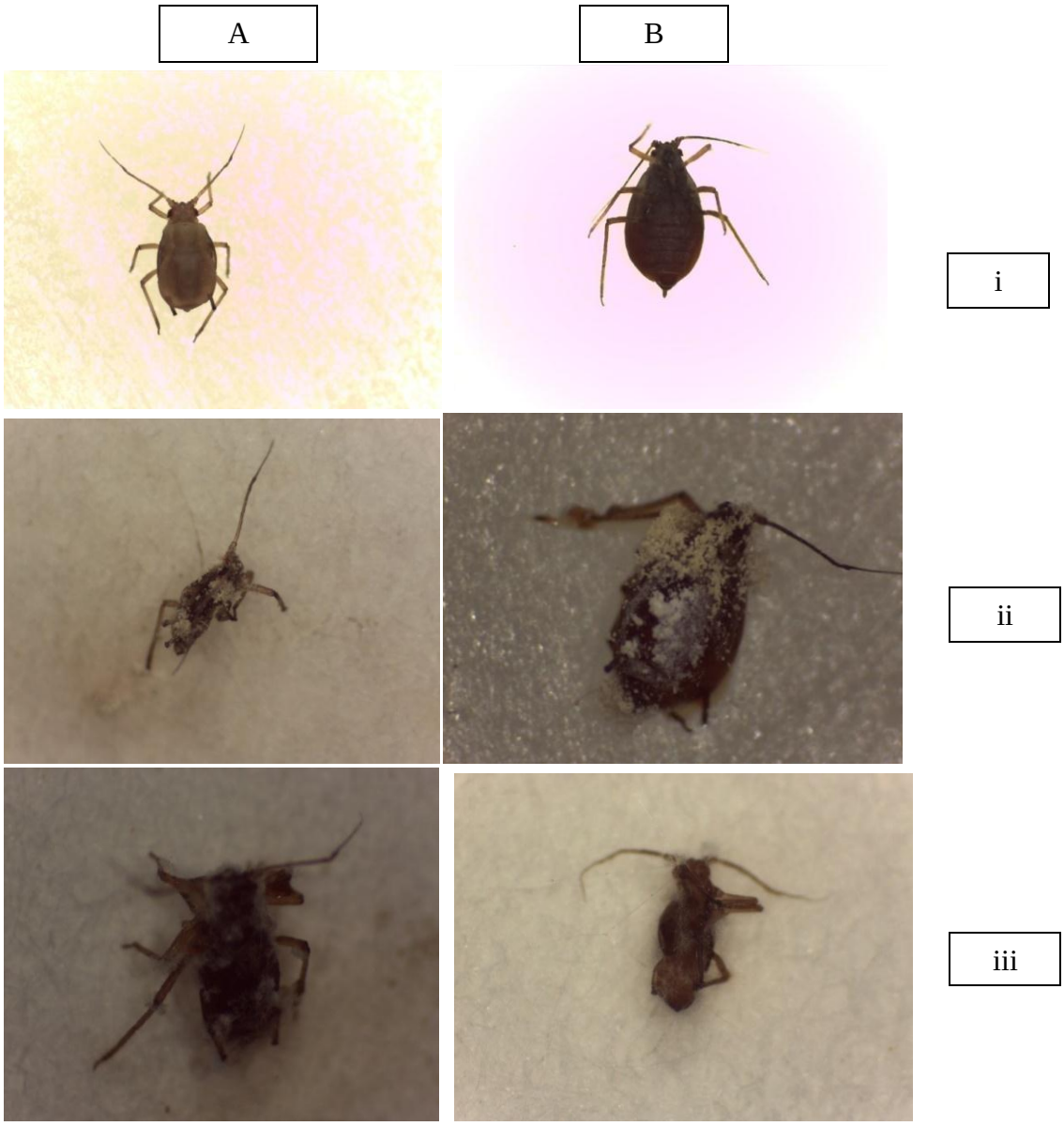

Figure 3.4 Photographs of mycosed cadavers of *M. dirhodum* (A) and *R. padi* (B) under laboratory conditions by fungi: *B. bassiana* (ii) and *M. anisopliae* (iii). (i) are the non-mycosed controls

3.3 Statistical Analysis

Aphid mortality was adjusted for control mortality using Abbot's formula (Abott, 1925). Analysis of variance (ANOVA) was performed on the percentage mortality data (transformed to arcsine (√ %) scale to normalize the variance. Means were separated using

Student-Newman-Keul's (SNK) test. LT_{90} and LT_{50} values were determined for each replicate using probit analysis method for correlation data and compared among themselves using ANOVA followed by mean separation by SNK. All analyses were carried out using the SAS version 9.1 (SAS Institute, 2003). The level of significance was set at 5% for all analyses to achieve the respective confidence intervals used to identify significant differences among the values of LT_{50} and LT_{90} for the different isolates of the fungi.

3.4 Results

Conidial viability of the isolates varied between 88 and 96% (Table 3.1). The results presented therefore indicate true differences in pathogenicity among the various isolates. Mortality in the controls for *R. padi* and *M. dirhodum* was approximately 8.3 % with a standard error (S.E.) of 1.4% and 11.9% with a S.E. of 1.5%, respectively.

All the fungal isolates were pathogenic to adult *R. padi,* causing mortalities ranging from 60.0 – 89.0% at 10 days post-treatment (Table 3.2). However, mortality varied between the fungal isolates with *M. anisopliae* isolates ICIPE 84, ICIPE 23, ICIPE 51 and ICIPE 78 being most virulent. The variations in susceptibility of adult *R. padi* and *M. dirhodum* and LT50 and LT90 values of isolates of *M. anisopliae* (n=14) and *B. bassiana* (n=6) are shown in Table 3.2 and 3.3, respectively. The LT_{50} values varied between 5.2 and 6.9 days while the LT_{90} values varied between 6.9 and 9.3 days. *M. anisopliae* isolates ICIPE 84, ICIPE 23, ICIPE 51 and ICIPE 31 had the shortest LT_{50} and LT_{90} values. The poorest performer among tested isolates was *B. bassiana* isolate NKOM with an LT_{50} value of 6.9 days and that took 9.3 days to induce 90% mortality (Table 3.2).

Results for screening *M. dirhodum* showed a similar pattern with ICIPE 84, ICIPE 51, ICIPE 23 and ICIPE 78 recording significantly higher mortalities than other isolates (Table 3). Mean mortalities of the four isolates ranged from 84.0 - 90.0%. The most virulent isolates induced 50% mortality within 4.6 – 5.1 days and 90 % mortality within 6.4 – 6.7 days. *Metarhizium anisopliae* isolates ICIPE 62, ICIPE 69 and ICIPE 273 had the longest LT_{50} values causing mortalities within 6.3 – 6.6 days (Table 3.3).

Table 3.2: Lethal Time (LT_{50} and LT_{90}) values of time-mortality responses of various isolates of *M. anisopliae* and *B. bassiana* against *R. padi* at a concentration of 10^7conidia/ ml.

Fungal Isolate	*Mean % Mortality*	*Mean % Mycoses*	*LT_{50} (Days)*	*LT_{90} (Days)*	*Slope*	$\chi 2$
M. anisopliae						
ICIPE 84	91.7 a	89.0 a	5.2(5.1 - 5.3)	6.9(6.8 - 7.0)	0.11	1337.6
ICIPE 23	90.9 ab	84.0 abc	5.2(5.2 - 5.3)	6.9(6.7 – 7.0)	0.12	1271.3
ICIPE 51	90.7 ab	85.0 ab	5.3(5.2 - 5.4)	6.9(6.8 - 7.0)	0.12	1287.4
ICIPE 31	84.3 abc	76.0 cde	5.7(5.6 - 5.7)	7.2(7.1 - 7.3)	0.13	1222.6
ICIPE 41	80.1 abcde	75.0 def	5.9(5.8 – 6.0)	7.6(7.5 - 7.7)	0.12	1296.3
ICIPE 07	77.8 abcde	60.0 i	6.0(5.9 - 6.1)	7.6(7.5 - 7.7)	0.14	1232.1
ICIPE 78	71.9 abcde	81.0 abcd	6.0(6.0 - 6.1)	7.8(7.7 - 7.9)	0.12	1342.9
ICIPE 25	83.5 abc	64.0 hi	6.1(6.0 - 6.2)	7.4(7.3 - 7.5)	0.18	1084.8
ICIPE 30	68.1 bcde	79.0 bcde	6.1(6.0 - 6.2)	8.0(7.9 - 8.1)	0.11	1369.6
ICIPE 62	70.6 abcde	61.0 i	6.3(6.2 - 6.3)	7.9(7.8 - 8.0)	0.14	1246.9
ICIPE 20	68.3 abcde	66.0 ghi	6.4(6.3 - 6.5)	7.7(7.6 - 7.8)	0.20	1062.4
ICIPE 69	58.4 de	60.0 i	6.5(6.4 - 6.6)	8.2(8.1 - 8.3)	0.14	1265.0
ICIPE 98	56.5 e	67.0 fghi	6.6(6.5 - 6.7)	8.1(8.0 - 8.2)	0.17	1134.5
ICIPE 60	59.7 de	67.0 fghi	6.6(6.6 - 6.7)	8.2(8.1 - 8.4)	0.15	1186.0
B. bassiana						
ICIPE 279	81.0 abcd	78.0 bcde	5.8(5.7 - 5.9)	7.3(7.2 - 7.4)	0.14	1221.3
ICIPE 271	77.6 abcde	71.0 efgh	5.9(5.8 – 6.0)	7.5(7.4 - 7.6)	0.13	1274.0
ICIPE 285	78.4 abcde	73.0 defg	6.0(5.9 - 6.1)	7.5(7.4 - 7.6)	0.14	1224.1
ICIPE 294	74.4 abcde	75.0 def	6.1(6.0 - 6.2)	7.6(7.5 - 7.7)	0.14	1231.7
ICIPE 273	61.5 cde	71.0 efgh	6.42(6.3 - 6.5)	7.9(7.8 - 8.0)	0.16	1188.8
Nkom	61.6 cde	80.0 bcd	6.87(6.8 – 7.0)	9.3(9.1 - 9.5)	0.10	1306.0
Means	74.4	73.1				
CV (%)	25.2	9.1				

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test. Values in parentheses signify 95% confidence limits of LT_{50} and LT_{90}.

Table 3.3: Lethal Time (LT_{50} and LT_{90}) values of time-mortality responses of various isolates of *M. anisopliae* and *B. bassiana* against *M. dirhodum* at a concentration of 10^7conidia/ ml.

Fungal Isolate	*Mean % Mortality*	*Mean % Mycoses*	*LT_{50} (Days)*	*LT_{90} (Days)*	*Slope*	$\chi 2$
M. anisopliae						
ICIPE 23	95.0 ab	88.0 abc	4.6(4.6 - 4.7)	6.4(6.3 - 6.5)	0.09	1341.9
ICIPE 84	96.7 a	84.0 abcd	4.8(4.8 - 4.9)	6.6(6.5 - 6.7)	0.10	1352.1
ICIPE 51	91.7 ab	90.0 a	5.1(5.0 - 5.1)	6.7(6.6 - 6.8)	0.11	1265.8
ICIPE 78	79.6 abcde	89.0 ab	5.2(5.1 - 5.3)	7.4(7.2 - 7.5)	0.08	1526.2
ICIPE 31	70.8 bcdef	78.0 def	5.6(5.6 - 5.7)	7.7(7.6 - 7.8)	0.09	1497.7
ICIPE 25	84.7 abcd	70.0 fg	5.7(5.6 - 5.8)	7.2(7.1 - 7.4)	0.13	1244.8
ICIPE 30	73.5 abcdef	80.0 cde	6.0(5.9 - 6.1)	7.8(7.6 - 7.9)	012	1349.6
ICIPE 20	79.6 abcde	60.0 h	6.1(6.1 - 6.2)	7.5(7.4 -7.6)	0.17	1128.3
ICIPE 60	70.8 bcdef	68.0 gh	6.2(6.1 - 6.2)	7.7(7.6 - 7.9)	0.14	1228.7
ICIPE 07	66.5 cdef	61.0 h	6.3(6.2 - 6.3)	7.8(7.7 - 7.9)	0.15	1222.5
ICIPE 98	71.7 bcdef	60.0 h	6.3(6.2 - 6.4)	7.7(7.6 - 7.8)	0.17	1146.1
ICIPE 62	64.4 cdef	68.0 gh	6.3(6.3 - 6.4)	8.0(7.9 - 8.2)	0.13	1310.4
ICIPE 41	59.2 ef	70.0 fg	6.4(6.4 - 6.5)	8.2(8.1 - 8.4)	0.13	1314.5
ICIPE 69	51.9 f	60.0 h	6.6(6.5 - 6.7)	8.2(8.0 - 8.3)	0.16	1185.7
B. bassiana						
ICIPE271	85.0 abcd	81.0 bcde	5.4(5.3 - 5.5)	7.2(7.1 - 7.3)	0.10	1383.5
ICIPE279	93.5 ab	80.0 cde	5.4(5.4 - 5.5)	7.0(6.8 - 7.0)	0.13	1225.6
ICIPE285	85.8 abc	78.0 def	5.7(5.6 - 5.7)	7.2(7.1 - 7.3)	0.14	1217.1
Nkom	75.9 abcdef	76.0 defg	6.0(5.9 - 6.0)	7.6(7.5 - 7.7)	0.13	1267.1
ICIPE294	72.5 abcdef	79.0 cde	6.2(6.1 - 6.3)	7.8(7.7 - 7.9)	0.15	1218.1
IC273	60.6 def	74.0 efg	6.4(6.3 - 6.5)	8.0(7.9 - 8.2)	0.14	1236.1
Means	76.5	74.7				
CV (%)	25.6	8.65				

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test. Values in parentheses signify 95% confidence limits of LT_{50} and LT_{90}.

3.5 Discussion

Entomopathogenic fungi have been observed to cause mortality in pest populations and thus investigated for their potential as biological control agents (Hesketh *et al*., 2008) or successfully developed as biocontrol agents against a number of different pests, including aphids (Shah and Pell, 2003; De Faria and Wraight, 2007). Conidial germination for all isolates varied from 86 to 93%. The results presented therefore indicate true differences in pathogenicity among the various isolates.

All the fungal isolates tested in this study were pathogenic to both *R. padi* and *M. dirhodum* in the laboratory, however, aphid mortality and LT_{50} values varied considerably between the isolates. Many studies have reported intraspecific variations in pathogenic activity of fungal pathogens on many groups of arthropod pests (Maniania and Fargues, 1984; Poprawski *et al*., 1985; Feng and Johnson, 1990; Ekesi *et al*., 1998; Mburu *et al*., 2009; Migiro *et al*., 2010). This underlines the importance of strain selection as observed by Soper and Ward (1981).

Contrary to the findings of these studies and those of Mburu *et al*., (2009) that M. *anisopliae* was better than *B. bassiana* in laboratory-based studies on mortalities against wheat aphids and termites respectively, Ibrahim *et al*. (2011) reported that calculated LC_{50} and LT_{50} values suggested that *B. bassiana* was more effective against both, *M. persicae* and *B. tabaci*, than *M. anisopliae.* The LT_{50} values obtained in this study for the best performing fungi (*M. anisopliae* isolates ICIPE 23, ICIPE 51 and ICIPE 84) against *R. padi* and *M. dirhodum*, ranged from 5.1 to 5.4 days and from 4.6 to 5.1 days for *R. padi* and *M. dirhodum* respectively. These time-mortality values were lower than 6.6 days at10^7spores ml^{-1} reported against the cowpea aphid by Saranya *et al*. (2010) and within the same range of that reported by Hesketh *et al*. (2008) who recorded a LT_{50} value of 5.5 days for *Metarhizium anisopliae* but at a higher concentration of 10^8 spores ml^{-1} against *Aphis fabae.* Lower values of LT_{50} have been demonstrated to lead to higher rates of infection, which indicates that the isolate has a higher virulence level (Facundo *et al*., 2001). A short period of lethal infection indicates a high level of pathogenicity or virulence of a pathogen and, in contrast, a long period of lethal infection indicates a low level of pathogenicity (Tanada and Kaya, 1993;

Fuxa and Richter, 2004). It is interesting to note that Mburu *et al*. (2009) reported that *M. anisopliae* isolate ICIPE 51 used during this study emerged as the most pathogenic among 15 *M. anisopliae* and 3 *B. bassiana* isolates tested against termites, *M. michaelseni*. In further agreement with the findings of this study, Mburu *et al*. (2009) classified *M. anisopliae* isolate ICIPE 69 as the least pathogenic among tested isolates. However, whereas *M. anisopliae* isolate ICIPE 60 showed good control against termite population, it was evidently inferior against aphid hosts in the laboratory trials.

The results of time-mortality laboratory study showed that three isolates identifiable as ICIPE 84, ICIPE 51 (EMBU 27) and ICIPE 23 (GM), registered the lowest values of LT_{50} and LT_{90}. Low time-mortality values have been demonstrated to lead to higher rates of infection, indicating higher virulence levels of the isolates. The three isolates were thus chosen as ideal candidates for further dose-mortality bioassays for development of microbial control of *R. padi* and *M. dirhodu*m.

CHAPTER FOUR

EFFECT OF PATHOGEN INOCULUM CONCENTRATION OF *METARHIZIUM ANISOPLIAE* (METCH) ISOLATES AGAINST *RHOPALOSIPHUM PADI* (LINNEAUS) AND *METOPOLOPHIUM DIRHODUM* (WALKER)

4.1 Introduction

Recent concerns about the hazardous effects of chemical pesticides to the environment and humans has encouraged scientists to consider finding more effective and safe control agents (Sezen *et al*., 2004; Muratoglu *et al*., 2011). Considering the adverse effect of insecticides, pest management through biological control is encouraged using predators, parasites and pathogens. Among the different microbial agents, entomopathogenic fungi (EPF) are gaining importance in pest control. More than 750 species of fungi are pathogenic to insects and many of them offer great potential for the management of sucking pests (Rabindra and Ramanujam, 2007). The potential of entomopathogenic fungi often varies among fungal species and strains.

Achieving or improving efficacy in microbial control can rely on choosing the best entomopathogen for a particular system. The most suitable entomopathogen from a variety of candidates can be selected simply by screening existing species and strains that possess superior desired traits such as virulence and environmental tolerance. New entomopathogens can be discovered through surveys and screened in parallel to existing strains; such surveys have been conducted extensively for entomopathogenic fungi (Leland *et al*., 2005; McGuire *et al*., 2005; Lubeck *et a*l., 2008). The screening process is often accomplished by first narrowing down the number of candidates in laboratory comparisons. Such comparisons have been made to find superior entomopathogen strains for numerous target pests (Shi and Feng, 2004; Shapiro-Ilan *et al.*, 2008a; Castrillo *et al.*, 2010). In previous screening experiments, three isolates of *M. anisopliae*, ICIPE 84, ICIPE 23 and ICIPE 51 demonstrated promise for use as potential biological control agents against *R. padi* and *M. dirhodum* by recording the lowest LT_{50} and LT_{90} values.

In addition to increasing the percentage of infected individuals in a given host population, pathogen density may also determine the number of propagules picked up by each host. Heavier doses of conidia have been reported to lead to earlier and / or higher host mortalities. This might lead to dead infected individuals liberating conidia earlier, further increasing the pathogen load in the environment. (Navon and Ascher, 2000). In the present study, the dose-response bioefficacy of the three selected isolates of *M. anisopliae* (Metsch.) Sorokin against *R. padi* and *M. dirhodum* was further evaluated under laboratory conditions.

4.2 Materials and Methods

4.2.1 Aphids and fungus

Metopolophium dirhodum and Rhopalosiphum padi colonies were separately maintained at ICIPE's screen house on potted caged "Mbuni" wheat variety, *Triticum aestivum* L. seedlings at a temperature of 26 ± 3°C, a photoperiod of 12:12 (L: D) and 70 – 80% relative humidity. Three-five days old nymphs were used in experiments. All the isolates in this study were obtained from the ICIPE culture collection germplasm. The isolates used were *M. anisopliae* (Metsch.) ICIPE 23 (GM), ICIPE 84 and ICIPE 51 (EMBU 27). (Table 4.1). These 3 were selected from 20 isolates for showing promise as microbial control agents against *M. dirhodum and R. padi* in previous screening experiments. (Murerwa *et al.*, 2014a).

Table 4.1: Origin and viability of three *Metarhizium anisopliae* isolates used for the dose-response bioassay against *Rhopalosiphum padi* and *Metopolophium dirhodum*

Species/Isolate/ Depositor's No.	ICIPE Accession No.	Origin of isolates, Locality, Country	% germination	Year of isolation
ICIPE 84	ICIPE 84	Sorghum, Kendu Bay, Kenya	86.0 ± 3.6	1989
GM	ICIPE 23	Niger	93.1 ± 2.2	1998
EMBU 27	ICIPE 51	Embu, Kenya	91.1 ± 3.7	2005

4.2.2 Conidial viability tests

The fungal isolates were cultured on Sabouraud dextrose agar (SDA) and incubated for 3 weeks at 26 ± 2°C. Conidia were harvested from the surface culture by scrapping. The inoculum was suspended in sterile distilled water with 0.05% Triton-100 in a glass bottle containing glass beads (3mm). The bottle was vortexed to produce a homogeneous conidial suspension. The spore concentration was quantified with the improved Neubauer haemocytometer. The viability of conidia was determined by spread-plating 0.1 ml of conidial suspension (titrated to 3 x 10^6 conidia ml^{-1}) on SDA plates. Sterile microscope cover slips were placed on each plate. The plates were incubated at 24-29^{0}C and examined after 20 h. Percent germination was determined by counting approximately 100 spores for each plate at X40 magnification. Each plate served as a replicate with six replications per isolate. In viability tests 86-93% of conidia germinated after 16 h (Table 4.1).

4.2.3 Application of *Metarhizium anisopliae*

Five serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made from the original (stock) spore suspension. The spore concentration of the last two dilutions was determined using an improved Neubauer haemocytometer slide. This was used to determine the spore concentration of the original spore suspension. The concentration was adjusted by dilution to get the respective spore suspensions of 1x 10^5, 3x 10^5, 1x 10^6, 3 x 10^6 and 1 x 10^7 conidia per ml. The protocol for inoculation remained as described in section 3.2.5 of the previous chapter.

4.3 Statistical analysis

The weighted mean time to death was calculated for each group of aphids infected with the same concentration of conidia after correcting for natural mortality (Abott, 1925) for each replicate as follows: The sum of the number of aphids dying of mycosis per day was multiplied by the time of each death, then divided by the total number of deaths due to the fungus at the end of the experiment. These values were transformed using square root (x + 1) transformation to stabilize variances and analyzed by a one-way ANOVA (SAS Institute, 2003). The level of significance was set at 5% for all analyses to achieve the respective

confidence intervals used to identify significant differences among values of LC_{50} and LC_{90} for the different isolates of the fungus.

4.4 Results

The highest concentrations of 1.0×10^7 spores/ml of *M. anisopliae* were most effective causing mortalities ranging from 89.0 – 97.0% and 91.7 – 98.9 % for *R. padi* and *M. dirhodum*, respectively at the end of experiment. Significant ($P<0.05$) differences in mycoses on cadavers were found between these and other concentrations of selected isolates in both aphid species. (*R. Padi*: F df (14, 60) = 5.68, P < 0.0001; *M. dirhodum:* Fdf (14, 60) = 3.13, p = 0.0011). The lowest mortalities for *R. padi* and *M. dirhodum* ranged from 34.0 – 42.2 % and 38.6 – 45.2 %, respectively and were recorded in concentrations of 1 $x10^5$ and 3 $x10^5$ in both aphid species. Mean percentage mortality and mycoses for *R. padi* and *M. dirhodum* at the end of experiment caused by different concentrations of each fungus is shown in Table 4.2.

Table 4.2: Effect of pathogen inoculum size of selected *M. anisopliae* isolates on mortality and mycosis of *R. padi* and *M. dirhodum*

Isolate	*Dose*	*Rhopalosiphum padi*		*Metopolophium dirhodum*	
		%Mortality ± S.E	Mean % Mycoses on cadavers	%Mortality ± S.E	Mean % Mycoses on cadavers
ICIPE 23	$1X10^5$	42.2 ± 4.5	78.7 e	45.2 ± 2.8	80.7 cd
ICIPE 23	$3X10^5$	50.5 ± 2.6	80.6 de	45.2 ± 3.8	81.7 cd
ICIPE 23	$1X10^6$	61.7 ± 3.0	85.5 bcd	63.1 ± 3.1	81.5 cd
ICIPE 23	$3X10^6$	68.0 ± 2.2	84.0 cde	74.7 ± 2.6	84.3 bc
ICIPE 23	$1X10^7$	87.7 ± 2.5	89.7 abc	96.8 ± 1.3	90.8 a
ICIPE 51	$1X10^5$	43.0 ± 2.5	84.0 cde	38.6 ± 4.3	83.2 cd
ICIPE 51	$3X10^5$	49.0 ± 1.0	83.6 cde	53.0 ± 3.1	81.6 cd
ICIPE 51	$1X10^6$	64.0 ± 1.9	84.2 cde	63.5 ± 1.7	84.6 bc
ICIPE 51	$3X10^6$	85.0 ± 2.7	91.6 ab	79.2 ± 2.8	92.5 a
ICIPE 51	$1X10^7$	97.0 ± 2.0	93.8 a	98.9 ± 1.1	94.9 a
ICIPE 84	$1X10^5$	34.0 ± 1.9	82.2 de	44.0 ± 4.9	78.0 d
ICIPE 84	$3X10^5$	41.0 ± 3.3	85.4 bcd	52.3 ± 5.6	82.1 cd
ICIPE 84	$1X10^6$	52.0 ± 2.6	86.5 bcd	61.0 ± 1.4	84.2 bc
ICIPE 84	$3X10^6$	58.0 ± 4.1	85.4 bcd	67.3 ± 2.3	82.7 cd
ICIPE 84	$1X10^7$	89.0 ± 1.9	88.7 abc	91.7 ± 3.1	89.4 ab

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test.

The LC_{50}s and LC_{90}s were estimated for the three isolates applied to *R. padi* and M. dirhodum. Results of the probit analysis indicate significant relationships between log dosage and probit mortality (Table 4.3). *R. padi*, isolate ICIPE 51 caused 50 and 90 percent mortality

at the lowest concentration of 2.42 x 10^5 and 5.00 x 10^6 spores ml^{-1} respectively. The least toxic concentration to induce 50 and 90 percent mortality was 6.19 x 10^5 and 3.61 x 10^7 spores ml^{-1}, respectively for isolate ICIPE 84. As shown in Table 4.3, there was no significant difference in the LC_{50} values for *M. dirhodum* and they ranged from 2.48 – 2.67 x 10^5 spores ml^{-1}. Isolate ICIPE 51 registered the least LC_{90} of 5.33 x 10^6 spores ml^{-1} which compared significantly with the highest LC_{90} of 2.16 x 10^7 spores ml^{-1} of isolate ICIPE 84.

Table 4.3: Lethal concentration (LC_{50}) values of dose-mortality responses of selected *M. anisopliae* isolates against *R. padi* and *M. dirhodum*

Species	Isolate	LC_{50}	95%Fiducial limits		LC_{90}	95%Fiducial limits	
R. padi	ICIPE 23	2.7 x 10^5	2.1 x 10^5	3.4 x 10^5	4.6 x 10^7	2.8 x 10^7	8.6 x 10^7
	ICIPE 51	2.4 x 10^5	2.0 x 10^5	2.8 x 10^5	6.5 x 10^6	5.0 x 10^6	8.7 x 10^6
	ICIPE 84	6.2 x 10^5	5.2 x 10^5	7.4 x 10^5	5.7 x 10^7	3.6 x 10^7	1.0 x 10^8
M. dirhodum	ICIPE 23	2.7 x 10^5	2.2 x 10^5	3.1 x 10^5	1.2 x 10^7	9.0 x 10^6	1.8 x 10^7
	ICIPE 51	2.6 x 10^5	2.2 x 10^5	3.1 x 10^5	6.9 x 10^6	5.3 x 10^6	9.3 x 10^6
	ICIPE 84	2.5 x 10^5	1.9 x 10^5	3.1 x 10^5	3.5 x 10^7	2.2 x 10^7	6.2 x 10^7

Failure of 95% confidence limits to overlap was used as the criteria for identifying significant differences among LC_{50} and LC_{90} values of selected isolates.

4.5 Discussion

The mortalities against *R. padi* and *M. dirhodum* were found to be directly proportional to the spore concentrations (Table 4.2) At the highest concentration of 10^7 spores ml^{-1}, *M. anisopliae* isolate ICIPE 51 gave appreciable reduction in population of *R. padi* and *M. dirhodum* showing 97.0 and 98.9 per cent, respectively. Ekesi *et al.* (2000) also got similar results with 93 per cent mortality of *Aphis craccivora* at 7^{th} day of treatment. Loureiro and Moino (2006) recorded 100 % mortality of *Myzus persicae* with *M. anisopliae* applied at 10^7 spores ml^{-1}. A progressive reduction in the mortality of aphids was observed with decreasing concentration. Saranya *et al*. (2010) observed that in lower concentrations mortality of

cowpea aphids ranged between 19.2 to 38.5 per cent. Recent studies also reported a well fit time–concentration–mortality model of ten *M. anisopliae* isolates against *M. persicae* (Shan and Feng, 2010). The foregoing indicates that at low spore concentration the time taken for multiplication may be prolonged resulting in a reduced control of the aphids. In addition to increasing the percentage of infected individuals in a given host population, the pathogen density may also determine the number of propagules picked up by each host. Heavier doses of conidia have been reported to lead to earlier and / or higher host mortalities. This might lead to dead infected individuals liberating conidia earlier, thereby further increasing the pathogen load in the environment. In general, however, with other entomopathogens, a minimal number of infective propagules are needed to pass through the portal of entry for infection to occur. (Navon and Ascher, 2000).

LC_{50} values for the most pathogenic *M. anisopliae* isolate ICIPE 51 of 2.4 x 10^5 and 2.6 x 10^5 spores ml^{-1} for *R. padi* and *M. dirhodum*, respectively obtained in the present study were lower than that reported by Saranya *et al*. (2010) for cowpea aphid (8.9 x 10^5 spores ml^{-1}), but higher than reported by Liu *et al.* (1999) for *B. bassiana* (1.2x10^4 spores ml^{-1} and Smitha (2007) for *Hirsutella* sp (5.2x10^4 spores ml^{-1}). Dose-response results indicated that ICIPE 51 outperformed isolates ICIPE 23 and ICIPE 84 by recording the lowest LC_{50} and LC_{90} values (Table 4.3). Virulence has always been one of the most important parameters considered for strain selection (Inglis et al. 2001). The difference in the LC_{50} values might be due to the difference in the virulence of fungal isolates and the host species. Differences in degrees of virulence of strains belonging to the same fungal species could also be attributed to genetic variations arising from specialization toward a determined host and the geographical distribution of the strains (Alves, 1998; Coates *et al*., 2002). In this case, isolates belonged to soil samples and insect hosts of different geographical origins (Table 3.1). Geographical and ecological features of the fungal isolates may explain the variation observed in this study (Meyling and Eilenberg 2007; Bischoff *et al*. 2009; Enkerli and Widmer 2010). For instance, *M. anisopliae* isolate ICIPE 51 originated from the Embu County, Kenya, whereas the ICIPE 23 originated from Niger.

Dose-response results indicated that ICIPE 51 outperformed isolates ICIPE 23 and ICIPE 84 by recording the lowest LC_{50} and LC_{90} values. Virulence has always been one of the most important parameters considered for strain selection (Inglis et al. 2001). It appears that *M. anisopliae* isolate ICIPE 51 can provide protection against more than one insect pest. Development of biocontrol agents to be used as myco-pesticides will play a significant role in sustainable development of agricultural practices resulting in reduction in health and environmental hazard caused by chemical pesticides. Experiments have shown *M. anisopliae* isolates to be non-pathogenic to natural enemies and beneficial soil insect (Jaronski *et al*., 1998; Thungrabeab and Tongma, 2007). There is no documented case where a fungal pathogen introduced for classical biological control of an insect pest caused substantial mortality to non-target species or had caused negative effect on human and animal health, or any significant impact on the environment (Hajek *et al*., 2003). No reports of resistance have been documented on entompathogenic fungi, a factor that makes them quite suitable for the control of aphids. Native pathogens present in natural pest population can be augmented by inoculation or inundation to establish epizootics (Shah and Pell, 2003).

Further studies are however required to validate these results in field experiments by testing the most virulent isolate, ICIPE 51 to determine its effect on various developmental stages of *R. padi* and *M. dirhodum*. Moreover, development of formulations and application methods for potentially effective fungi is also necessary, because the humidity and temperature of a greenhouse or field air is not always favourable for fungal infection when compared with the optimized laboratory conditions.

CHAPTER FIVE

PATHOGENICITY OF *METARHIZIUM ANISOPLIAE* ISOLATE ICIPE 51 AGAINST DIFFERENT DEVELOPMENTAL STAGES OF *RHOPALOSIPHUM PADI* (LINNEAUS) AND *METOPOLOPHIUM DIRHODUM* (WALKER)

5.1 Introduction

Bird-Cherry oat aphid, *Rhopalosiphum padi* (Linnaeus) and Rose Grain aphid, *Metopolophium dirhodum* (Walker) pose a serious threat to bread wheat growers in Kenya. Both nymphs and adults suck plant sap and cause serious damage right from the seedling to maturity stage. In addition, these aphids also serve as the most efficient vectors of viral diseases including BYDV (Riedell *et al*., 2003; Jiménez-Martínez *et al*., 2004; Fabre *et al*., 2006; Borer *et al*., 2009, Anne Wangai, personal communication).

Insecticides of synthetic origin have been used to manage insect pests for more than 50 years (Charnley and Collins, 2007). However, due to adverse effects of insecticides to the environment, their rational use is being advocated. Entomopathogens as biocontrol agents have several advantages when compared with conventional insecticides. These include low cost, high efficiency, safety for beneficial organisms, reduction of residues in the environment and increased biodiversity in human managed ecosystems (Lacey *et al*., 2001). Fungal biological control agents have demonstrated efficacy against a wide range of insect pest species (Purwar and Sachan, 2005; Lin *et al*., 2007; Amer *et al*., 2008).

Cereal infesting aphids are multivoltine pests and individuals in all developmental stages are usually present on an infested wheat crop. (Helmut and Richard, 2007). A pathogen that is able to cause infection and kill more than one developmental stage of its host may be better able to suppress the population of such a host. In studying mycoses, most workers have used either only adult aphids or individuals of unspecified stages. When considering the effectiveness of an entomopathogen against a host with a high reproductive potential, estimation of adult mortality alone may not be satisfactory. This caution should be extended to advice against testing pathogenicity against only one developmental stage. Such

information may be important in assessing establishment and impact of an exotic pathogen on its host. In this case, evaluating the effectiveness of a fungal pathogen against one nymphal stage could underestimate the field efficacy of *Metarhizium anisopliae*.

Insect susceptibility to fungal infection has been reported to be affected by a number of factors, such as the properties of the pathogen population, the host population as well as environmental conditions (Inglis *et al*., 2001). Among the host factors, host species, host age, the developmental stage and sex have been reported to affect host susceptibility to entomopathogenic fungi. According to Butt *et al*. (2001), an understanding of host properties that influence susceptibility to infection is important in the development of management tactics and will enable the optimization of the impact of biological control agents.

No studies have been undertaken so far to investigate the differential susceptibility of different developmental stages of *R. padi* (L.) and *M. dirhodum* (Walker) to fungal infection. This study, investigated the influence of the developmental stages of two aphid species on their susceptibility to *M. anisopliae* isolate ICIPE 51 and the implications for biological control of these important pests of bread wheat in Kenya.

5.2 Materials and Methods

5.2.1 Rearing of Uniform Aged Aphids

Metopolophium dirhodum and *Rhopalosiphum padi* were reared on wheat plants, *Triticum aestivum.*, variety "Mbuni" in ventilated Plexiglas cages (60 x 35 x 70 cm) at temperatures between 24-28^{0}C, 60-70% relative humidity (RH) and a photoperiod of 12 :12 h (light : dark) in a rearing room at the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. The initial culture originated from aphids collected from Njoro, Kenya in 2006.

To obtain the different developmental stages for the experiments, adult aphids were collected from the aphid culture and put on fresh leaf discs placed on wet cotton wool in Petri dishes. The inoculated aphids reproduced parthenogenetically. Newly emerged one-day old first instar nymphs were transferred to new leaf discs and thereafter leaf discs were changed every 4 days.

The rearing was carried out in such a way that different developmental stages were obtained at the same time so that treatments could be performed concomitantly.

5.2.2 Fungal pathogen

Metarhizium anisopliae isolate ICIPE 51 was used in the study. It was sourced from the *icipe* Arthropod Germplasm Centre and was selected because of its virulence to *M. dirhodum* and *R. padi* demonstrated in earlier screening and dose-response experiments. (Murerwa *et al.*, 2014a, b). The fungus was grown for 21 days on Sabouraud dextrose agar (SDA) plates at $26^0C \pm 2^0C$. Conidia were harvested by scrapping the surface using a sterile rubber. Inocula were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads. Conidial suspensions were vortexed for 5 min to produce a homogenous suspension. Spore concentrations were determined using a haemocytometer. Viability of conidia was determined before each bioassay by spread-plating 0.1 ml of conidial suspension titrated at 3.0×10^6 conidia/ml on SDA plates. Sterile microscopic cover slip was placed on each plate and plates were incubated at 26 ± 2°C and examined after 15 h. Percentage germination was determined from 100-spore counts. Each plate was replicated four times. Over 94 % of conidia germinated in all the tests.

5.2.3 Inoculation of the developmental stages

Nymphs aged 0-2 days, 3-4 days and adults (5-7 days old) were used in the bioassays. Both sides of fresh wheat leaves were sprayed with 10 ml of conidial suspension using Burgerjon's spray tower and allowed to dry for 20 min. Aphids were then transferred to the leaf discs in Petri dishes (90 mm diameter) using a camel hair brush. Concentrations of 1.0×10^6, 3.0×10^6 and 1.0×10^7 conidia/ ml were used for each developmental stage. Control lots were treated with sterile distilled water containing 0.05% Triton X-100. Test-aphids were exposed to treated wheat leaf discs for 4 days, after which treated discs were removed and replaced with fresh and untreated leaf discs. Aphids were maintained in an incubator at $26^0C \pm 2^0C$ and 70-80% RH. Mortality was recorded daily for 10 days. Dead aphids were transferred to Petri dishes lined with moist filter paper to allow the growth of the fungus on the surface of the cadavers. Mycosis was confirmed by microscopic examination. Treatments consisted of 20 aphids each and were replicated 5 times.

5.3 Statistical analysis

Percentage mortality was normalized through angular transformation after correcting for natural mortality (Abott, 1925). Mortality rates were separated across treatments using the ANOVA procedure of SAS (SAS Institute, 2003). Mean values were separated using LSD at 0.05 level.

5.4 Results

In the viability test more than 94% of spores germinated. Table 5.1 shows the mortality caused by *M. anisopliae* isolate ICIPE 51 at different developmental stages among the two aphid species. There were significant differences among both aphid species observed in mortalities of all nymphal instars and adults ($P < 0.05$). 3-4 day-old nymphs were significantly more susceptible than 0-2 day-old nymphs. The 5–7 day old adults were the most susceptible stage to the fungus, with 40 and 33% mortality against *M. dirhodum* and *R. padi*, respectively as compared to 25 and 20% for *M. dirhodum* and *R. padi*, respectively registered among 0-2 day-old nymphs.

Table 5.1: Mean percent mortality of different nymphal instars and adults of *R. padi* and *M. dirhodum* treated with *M. anisopliae* isolate ICIPE 51

Stage	Mean Mortality (%)	
	M. dirhodum	*R. padi*
0-2 day-old nymphs	24.5c	19.8c
3-4 day-old nymphs	28.6b	26.3b
5-7 days old adults	40.3a	32.9a

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test.

There were differences in aphid mortality among all stages with increasing concentration of *M. anisopliae* isolate ICIPE 51 and these differences were statistically significant ($P < 0.05$). The lowest mortalities for *M. dirhodum* and *R. padi* was 19 and 16%, respectively recorded at 1 X 10^6 spores ml^{-1} among the 0-2 day-old nymphs while the highest mortalities for *M. dirhodum* and *R. padi* was 51 and 44% respectively registered at 1 X 10^7 spores ml^{-1} among the 5-7 days old adults. Percent mortality of different nymphal instars of *M. dirhodum* and *R. padi* treated with different concentrations of *M. anisopliae* isolate ICIPE 51 is shown in Table 5.2.

Table 5.2: Mean percent mortality of different nymphal instars and adults of aphids treated with different concentrations of *M. anisopliae* isolate ICIPE 51

Stage	Mean Mortality (%)					
	Dose (Conidia/ml)					
	1 X 10^6		3 X 10^6		1 X 10^7	
	M. dirhodum	*R. padi*	*M. dirhodum*	*R. padi*	*M. dirhodum*	*R. padi*
0-2 day-old nymphs	19.1c	15.9b	25.2c	20.2c	29.3c	23.4c
3-4 day-old nymphs	20.4b	23.1a	28.5b	25.3b	36.8b	30.6b
5-7 days old adults	30.7a	23.5a	38.8a	31.1a	51.3a	44.2a

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test.

M. anisopliae isolate ICIPE 51 was able to infect 3 and 4 day-old nymphs and 5-7 days old adults 48 hours after treatment whereas 0-2 day-old nymphs recorded mortality after 72 hours. 5-7 days old adults were the most susceptible taking between 5 - 6 days to register 50% mortality as compared to the 0-2 day-old nymphs which took the longest time of about 8 days. At the end of experiment, the lowest mortality of 75% and highest mortality of 91% were observed among the 0-2 day-old nymphs and 5-7 days old adults respectively (Table 5.3).

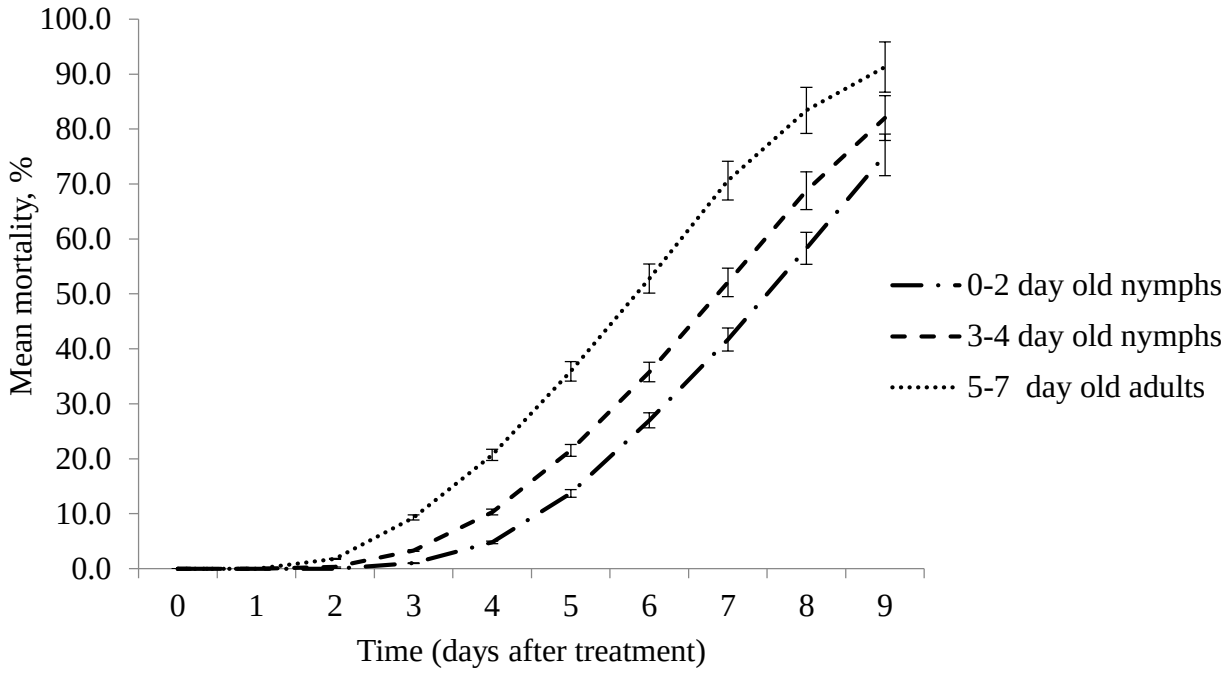

Figure 5.1 Effect of time on percent mortality of different nymphal instars and adults of *R. padi* and *M. dirhodum* treated with *M. anisopliae* isolate ICIPE 51

5.5 Discussion

Numerous studies indicate that aphids are susceptible to infection by diverse species of entomopathogenic fungi including *M. anisopliae* (Shan and Feng, 2010; Ibrahim *et al*., 2011). *Metarhizium anisopliae* isolate ICIPE 51 had pathogenic effects against *R. padi* and *M. dirhodum* although the latter was more susceptible with significant differences in mortality observed in all nymphal instars and adults. There was a progressive significant increase in susceptibility among both aphid species with increasing aphid age with the highest mortalities recorded among 5-7 days old adults.

There is little information on the effects of *M. anisopliae* on developmental stages of either *R. padi* or *M. dirhodum*. However, it is possible to make comparisons with other insects. In this study, adult aphids were more susceptible than the immature 0 – 4 day old nymphs and this agrees with observation of Lopes and Alves (2011) that demonstrated adults of *Blattella germanica* (L.) (Blattodea: Blattellidae) were more susceptible to *M. anisopliae* infection than nymphs. Likewise, according to Romaña and Fargues (1992), the older larvae of

Melolontha melolontha (L.) (Coleoptera: Scarabaeidae) were clearly more susceptible to *Beauveria brongniartii* than the younger larval instars. Similar results were reported from *Tetranychus urticae* (Saenz-de-Cabez Irigaray *et al*. 2003) with *B. bassiana*. This could explain the higher infection rate by *Neozygites floridana* in field-collected adults of *T. urticae* and *Halotydeus destructor* (Tucker) (Acarina, Penthaleidae) than in immature stages (Ridsill-Smith and Annells 1997).

In contrast, Haji Allahverdi Pour *et al*. (2008) demonstrated that fifth instar nymphs of Sunn pest were more susceptible to *B. bassiana* than adults. The foregoing reinforces an earlier observation by Ferron (1985) that relative susceptibility of different development stages of a host depends on the host species and on the fungal isolate. Ekesi and Maniania (2000) reported moulting to be an important factor in arthropod resistance to fungal infection, especially in arthropods with short ecdysis intervals. If the host is in an immature stage, molting could reduce the effectiveness of the fungal entomopathogen, in part owing to the shedding of conidia attached to the molted cuticle (Luz *et al.,* 2003). In this study, germinated and ungerminated conidia were observed on the exuviae of *R. padi* and *M. dirhodum* following infection with *M. anisopliae*. It is probable the fungal inoculum was shed off with the exuvium following ecdysis leading to differential susceptibility observed in different nymphal stages and specifically the apparent decreased susceptibility of the immature aphid stages. It is also possible that enhanced susceptibility of adult aphids could be attributed to observed increased mobility across leaf surfaces as compared to the less active immature stages thereby increasing chances of contact of relatively larger adult aphids with multiple fungal inocula.

Mortality in all stages was dose-dependent, with the highest mortality occurring at 10^7 conidia/ml. Comparable results were reported on *T. urticae* with *B. bassiana* (Senz-de-Cabezón Irigaray *et al*., 2003). Similar dose-mortality responses on different developmental stages have also been reported on many other arthropod pests (Feng *et al.,* 1985; Ekesi and Maniania, 2000). According to the results, high doses and long periods are required for the isolates to cause satisfactory levels of mortality.

In conclusion, this study demonstrated pathogenicity of *M. anisopliae* isolate ICIPE 51 against *R. padi* and *M. dirhodum* under controlled laboratory conditions. Virulence for all stages was dose-dependent and mortality increased with host aphids' maturity and over time. These results showed that *M. anisopliae* isolate ICIPE 51 could be a viable alternative to control *R. padi* and *M. dirhodum* in bread wheat.

CHAPTER SIX

EFFECTS OF *METARHIZIUM ANISOPLIAE* (METCH) ISOLATE ICIPE 51 INFECTION ON MORTALITY AND FECUNDITY OF *RHOPALOSIPHUM PADI AND METOPOLOPHIUM DIRHODUM*

6.1 Introduction

Bird cherry-oat aphid, *Rhopalosiphum padi* and the rose-grain aphid, *Metopolophium dirhodum* can reproduce parthenogenetically and viviparously to develop large populations in a short space of time (Blackman and Eastop 2000). In addition to their direct involvement as virus transmitters, aphids have large reproductive rates and their wide range of host plants mean that they are difficult to control. Selective aphicides have been used to reduce populations to below damage threshold levels (Ibrahim *et al*., 2011). However, concern about the hazardous effect of chemical pesticides on the environment and human has encouraged scientists to consider finding more effective and safe control agents (Sezen *et al*., 2004; Muratoglu *et al*., 2011). Entomopathogenic fungi are important in the natural regulation of many insect pests and pest populations are often decimated in widespread epizootics. They normally invade via the external cuticle and need not be ingested to initiate disease. This makes them prime candidates for use against plant sucking insects (Barta and Cagan, 2006).

Arthropod mortality, fecundity and fertility are affected by many factors including pathogens. It has been demonstrated that entomopathogenic fungi may cause sub-lethal reproductive effects on target individuals that can have important implications for the population dynamics of the host and contribute to the status of the target insect as a pest (Arthurs and Thomas, 2000; Blandford and Thomas, 2001; Mulock and Chandler, 2001; Quesada-Moraga *et al*., 2004). The reduction of the reproductive potential of *M. dirhodum* and *R. padi* adults that are fungally challenged during oviposition may contribute to the overall efficacy of the treatment. Despite many reports on the suppression of aphid populations as a consequence of fungal epizootics in the field (Pell *et al*., 2001) little is known about the extent to which entomopathogenic fungi affect the fecundity of the host aphid (Xu and Feng, 2002). Sub-lethal side-effects of the infection process may also result in a decrease in the number and of nymphs produced (Baverstock *et al*., 2006)

Results from preliminary screening and dose-response greenhouse experiments indicated that of *Metarhizium anisopliae* (Metschnikoff) Sorokin *isolate*, ICIPE51 holds potential for management of *R. padi* and *M. dirhodum*. (Murerwa *et al.*, 2014a, b). In the present study, the effects of fungal infection on fecundity and intrinsic rate of natural increase of *R. padi* and *M. dirhodum* were investigated.

6.2 Materials and Methods

6.2.1 Aphids and Fungal Pathogen

The aphid stock was obtained and maintained as described in sections 3.2.2. Conidial suspensions used in this experiment were of *M. anisopliae* isolate ICIPE 51 which had demonstrated high pathogenicity against *R. padi* and *M. dirhodum* in preliminary screening experiments. The protocol of its preparation and maintenance were as described in Chapter three sections 3.2.3. Germination and mycoses tests were performed as described in sections 4.2.1 of Chapter four.

6.2.2 Laboratory Bioassays

Five replicates of three conidial concentrations (1.0×10^6, 3.0×10^6 and 1.0×10^7 conidia / ml) were prepared as described in section 4.2.2 and inoculated onto groups each containing about 40-45 apterous adult aphids. This total included extra aphids to ensure that each treatment had 30 live aphids after being treated with a conidial suspension using the protocol described in section 3.2.5 in Chapter three. The protocol of handling the control groups remained as described in Chapter three, section 3.2.5. Treated aphids were transferred to a leaf in an assay cell, one aphid per cell. Each assay cell consisted of a 60-mm transparent plastic Petri dish containing a 5-cm length of wheat leaf from a greenhouse-grown plant (2-3 wks old) with the ends contacting bands of water-soaked, sterile cotton. The assay cells were maintained in the ventilated Plexiglas cages. The cotton wool in the Petri dishes was saturated daily with water and every 3 - 5 days aphids were transferred to new leaf disks. New born nymphs were removed after counting. The treated aphids were observed daily for 7 days to record mortality and fecundity.

6.3 Statistical analysis

Differences in fecundity and intrinsic rate of increase were tested by analysis of variance (Anova). If significant differences were detected, multiple comparisons were made using the LSD test (p=0.05). The intrinsic rate of natural increase (r_m) was calculated using the following formula as described by Wyatt and White (1977):

$$r_m = \frac{0.74(\ln Md)}{d}$$

where, Md is the number of nymphs produced over a period of time equal to that of the entire pre-reproductive period (d). This formula gives a good estimate of population growth rates in aphids (Dixon *et al*., 1993)

6.4 Results

6.4.1 Effect of pathogen inoculum concentration

Table 6.1 shows that the maximum fecundity in *M. dirhodum* and *R. padi* was 1.8 and 2.0 nymphs per aphid respectively observed at the lowest concentration of 1 X 10^6 spores ml^{-1}. Both aphids species were significantly less fecund at 1 X 10^7 ml^{-1}, registering 1.2 and 1.4 nymphs per aphid for *M. dirhodum* and *R. padi*, respectively. There was no significant difference in fecundity among both aphid species between the control and 1 X 10^6 spores ml^{-1} treatments. *M. dirhodum* was significantly less fecund than *R. padi* at all tested concentrations. The intrinsic rate of natural increase (rm) was different among the aphid species as well as among the treatments ($P < 0.05$). The rm value was the highest at 1 X 10^6 spores ml^{-1} (0.49 and 0.55 nymphs per aphid d^{-1} for *M. dirhodum* and *R. padi*, respectively) as compared to the lowest value of rm at 1 X 10^7 spores ml^{-1} (0.40 and 0.47 nymphs per aphid d^{-1} for *M. dirhodum* and *R. padi* respectively).

Table 6.1: Effect of dose on fecundity and intrinsic rate of increase of *M. dirhodum* and *R. padi* infection with *M. anisopliae* isolate ICIPE 51

Treatment	Fecundity		Intrinsic Rate of Increase ($_{rm}$), %	
	M. dirhodum	*R. padi*	*M. dirhodum*	*R. padi*
Control	1.8a	2.0a	0.49a	0.54a
1 X 10^6	1.8a	2.0a	0.49a	0.55a
3 X 10^6	1.6b	1.7b	0.47a	0.48b
1 X 10^7	1.2c	1.4c	0.40b	0.47b

Means followed by the same letter in a column are not significantly different ($p > 0.05$) using SNK test.

6.4.2 Effect of time on fecundity and intrinsic rate of increase of *R. padi* and *M. dirhodum*

There was a general progressive decline in fecundity over time in both aphid species (Table 6.2). Fecundity in the first 2 days among both species was more than 3 nymphs/aphid. Thereafter, fecundity at 4 days and 7 days post treatment reduced significantly and respectively to 1.5 and 0.1 nymphs/aphid and 1.8 and 0.1 nymphs/aphid for *M. dirhodum* and *R. padi* respectively.

The highest intrinsic rate of increase (rm) was recorded during the first day (0.82 and 0.91 nymphs/aphid/day for *M. dirhodum* and *R. padi*, respectively) while the lowest (0.20 and 0.26 nymphs/aphid/day for *M. dirhodum* and *R. padi*, respectively) was recorded on the seventh day.

Table 6.2: Effect of Time on fecundity and intrinsic rate of increase of *M. dirhodum* and *R. padi* infected with *M. anisopliae* isolate ICIPE 51

Days	Fecundity				Intrinsic Rate of Increase ($_{rm}$), %			
	M. dirhodum		*R. padi*		*M. dirhodum*		*R. padi*	
	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
1	3.0	3.3	3.5	3.7	0.82	0.87	0.91	0.96
2	3.1	3.5	3.2	3.7	0.67	0.71	0.69	0.74
3	2.6	2.7	2.8	3.0	0.53	0.56	0.55	0.58
4	1.5	1.9	1.8	2.1	0.43	0.45	0.45	0.46
5	0.7	0.9	1.0	1.2	0.30	0.33	0.36	0.40
6	0.3	0.5	0.3	0.5	0.23	0.28	0.30	0.33
7	0.1	0.1	0.1	0.2	0.20	0.25	0.26	0.29
LSD	0.1				0.02			
CV	19.9				10.8			

6.5 Discussion

Studies assessing the alarm response of pea aphids infected with either *P. neoaphidis* or *B. bassiana* support the hypothesis that host-specific fungi modify the behavior of the host whereas more generalist fungi do not (Roy *et al.*, 2005). Pathogen and host fitness are directly dependent on the number of viable offspring produced and it is predicted that both will be adopting strategies to maximize reproductive output. Many studies have demonstrated that a reduction in host fecundity can increase pathogen fitness as host resources such as energy are used by the pathogen for conidia production rather than by the host for reproductive output (Xu and Feng, 2002). In this study, both *R. padi* and *M. dirhodum* infected by *M. anisopliae* sustained an increase in reproductive output in response to early stages of infection to possibly ensure that part of their reproductive potential is realized. This

may also benefit the pathogen through ensuring the continuation of a susceptible host population (Blanford and Thomas, 2001).

Thereafter, both aphid species suffered a rapid and sustained decline in nymph production suggesting that resources were directed for the benefit of the pathogen, thereby supporting the above stated hypothesis. In contrast other studies have suggested that the pea aphid, *Acyrthosiphon pisum* aphids infected by *P. neoaphidis* registered fast and sustained decline in fecundity (Baverstock *et al*., 2006). The increase in fecundity followed by a reduction period may be a result of the host diverting resources to reproduction as a defense strategy to increase fitness. The subsequent reduction in fecundity observed 5 days post inoculation may be the outcome of an incidental process in which the indiscriminate invasion of host tissues and production of secondary metabolites interferes with nymph production. These hypotheses require further exploration.

It was noted that *M. anisopliae* isolate ICIPE 51 infection led to significant reduction of the host aphid's progeny in both species. Low levels of inocula (10^6 conidia/ml) of the entomopathogen appeared to have no significant effect on aphids' fecundity and intrinsic rate of increase. Baverstock *et al*. (2006) observed that infection of the pea aphid, *Acyrthosiphon pisum* by either *P. neoaphidis* or *B. bassiana* reduced the number of nymphs produced within 24 h of inoculation and over the entire infection period compared to uninfected aphids. However, infection for 24 or 72 h did not alter the intrinsic rate of increase of the host aphid. Similar results to this study were observed in the reproductive output of *Tutta absoluta* (Pires *et al*., 2008), using doses of 10^7 conidia ml^{-1} of *M. anisopliae* and *Diuraphis noxia* (Wang and Knudsen, 1993) using *B. bassiana.* Other studies that have shown comparable results on this topic include *Cylas puncticollis* (Ondiaka *et* al., 2008), *Anoplophora glabripennis* (Hajek *et al*, 2008) and *Megalurothrips sjostedti* (Ekesi and Maniania, 2000).

Metarhizium anisopliae isolate ICIPE 51 demonstrated pathogenicity against *R. padi* and *M. dirhodum* under controlled laboratory conditions. Virulence was dose-dependent and mortality increased with time. Low doses of the isolate do not affect pre-lethal reproductive effects, such as fecundity and intrinsic rate of increase. Both aphid species were significantly

more fecund in their early adulthood, suggesting the stage as ideal for biopesticide management intervention. On the other hand, it should be considered that laboratory and greenhouse bioassays are usually conducted under optimal conditions for fungal growth (high humidity and constant temperatures and photoperiods), which are obviously very different from the environmental conditions that will be encountered in the field (Butt and Goettel, 2000). Hence, additional research at field conditions to further evaluate and consolidate present findings regarding the biopesticide potential of *M. anisopliae* isolate ICIPE 51 would be necessary.

CHAPTER SEVEN

EFFECT OF DIFFERENT FORMULATIONS OF *METARHIZIUM ANISOPLIAE* (METCH) ISOLATE ICIPE 51 ON EFFICACY AGAINST *RHOPALOSIPHUM PADI* AND *METOPOLOPHIUM DIRHODUM*

7.1 Introduction

Different cereal aphid species occur on wheat fields causing serious yield losses through their direct and indirect damage (Ehsan-ul-Haq, 2003; Nyaanga *et al*., 2006; Van Emden and Harrington, 2007; Lapierre and Hariri, 2008). A major problem facing the bread wheat industry in Kenya is that most commercial varieties are susceptible to bird Cherry-oat aphid, *Rhopalosiphum padi* and the rose-grain aphid, *Metopolophium dirhodum.* Overuse of chemical insecticides against insect pests has had a negative impact on the environment (Lomer *et al*., 2001). It is thus necessary to develop alternative means to sustainable aphid control.

Studies of biodiversity in agro ecosystems and the delivery of ecosystem services to agricultural products have usually ignored the contribution of entomopathogens in the regulation of pest populations (Tschamtke *et al*., 2005). In recent years, crop protection based on biological control of crop pests with microbial pathogens like viruses, bacteria, fungi and nematodes has been recognized as a valuable tool in pest management (Rao *et al*., 2006; Anand *et al*., 2009). Various entomopathogenic fungi such as *Lecanicillium* sp. (Jung *et al*., 2006; Ownley, 2010), *Beauveria bassiana* (Quesada-Moraga *et al*., 2006; Sivasundaram *et al*., 2007 and Vega *et al*., 2010) and *Metarhizium anisopliae* (Ansari *et al*., 2007; Bukhari *et al*., 2010; Chandler and Davidson, 2005; Dong *et al*., 2007) have been effectively used to control aphids, lepidopteran larvae and other pests.

In addition to different species and strains of entomopathogenic fungi, different formulations and application methods have also been evaluated (Wraight *et al*., 2000; Wraight and Ramos, 2002; Feng *et al*., 2004a, b; Pu *et al*., 2005). Different formulations and application methods have been tested to evaluate the efficacy of the entomopathogenic fungi. De Faria and Wraight (2007) listed eleven different fungal formulation types employed to apply

entomopathogenic fungi against pests. The importance of verifying laboratory efficacy in the field cannot be overemphasized. An entomopathogen that shows high virulence in the controlled environment of a laboratory could fail to suppress the target pest in the field owing to various biotic or abiotic factors that render the organism incompatible. A lack of understanding of the biological and ecological constraints required for pathogen persistence and proliferation in the environment is likely to lead to a discrepancy between laboratory and field efficacy (Hu and St. Leger, 2002, Bruck, 2005; Bruck, 2010).

Successful use of entomopathogenic fungi as microbial control agents ultimately depends on the use of the right propagule, formulated in an optimum fashion and applied at the right time to a susceptible host. The formulation of entomopathogenic fungi against *R. padi* and *M. dirhodum* has not been studied previously in Kenya. To develop such formulations, an understanding of their efficacy on the target insects is necessary. The present study sought to search for a fungal formulation that may provide effective control of bird cherry-oat aphid and rose-grain aphid under field conditions at Njoro, Kenya. Three formulations (oil, sticker and water) of M. *anisopliae* isolate ICIPE 51 were compared together with Karate, a conventional aphicide widely used by local farmers in the region. (Macharia Munene, KALRO, pers. Comm). The objective was to establish an alternative strategy for *R. padi* and *M. dirhodum* control that facilitates applications of fungal formulations to bread wheat crop than the chemical, which encounters increasing resistance to aphids despite minor effects on their natural enemies (Prischmann et al., 2005). Specifically the study sought to:

- Determine the field efficacy of *M. anisopliae* isolate ICIPE 51 application in three formulations (oil, sticker and water) against *R. padi* and *M. dirhodum.*
- Determine the effect of three formulations of the fungus on the fecundity and intrinsic rate of increase of *R. padi* and *M. dirhodum.*
- Determine the pathogenic potential of the entomopathogenic formulations when applied before (Pre) and after (Post) infestation of bread wheat with *R. padi* and *M. dirhodum.*

7.2 Materials and Methods

7.2.1 Study Site description

The experiments were carried out in 2011 and 2012 at a greenhouse and research field in Egerton University. Egerton University is situated in Njoro town (0^0 23'S and 35^0 35'E) of Nakuru county and lies at an altitude of 2265 m a.s.l in Lower Highland (LH2 – LH3) agro ecological zone and has a sub-humid modified tropical climate. The annual average rainfall is 931mm, mean temperature ranges between 16 – 19.1^{0}C and mean maximum and minimum temperature are 22.7^{0}C and 7.9^{0}C, respectively. Soils are mollic andosols (Jaetzold and Schmidt, 1983).

7.2.2 Rearing of aphids

M. dirhodum and R. padi were reared and maintained on wheat plants, *Triticum aestivum*, variety "Mbuni" derived from clean wheat seeds provided by the plant breeding section of Kenya Agricultural Research Institute (KALRO-Njoro) in Jiffy pots (7cm x 7cm) filled with sterilized soil in ventilated Plexiglas cages (60 x 35 x 70 cm) at temperatures between 24-28^{0}C , 60-70% relative humidity (RH) and a photoperiod of 12 :12 h (light : dark) in a greenhouse at Egerton University.

7.2.3 Source, culture and preparation of fungal suspension

Metarhizium anisopliae isolate ICIPE51 was obtained from *icipe's* collection of Entomopathogenic Fungal Cultures and was selected for its superior potency in biocontrol *of R. padi* and *M. dirhodum* from preliminary screening and dose-response laboratory experiments. (Murerwa *et al.*, 2014a, b). It was cultured on Sabouraud dextrose agar (SDA) plates and incubated at 25 ± 1°C. Conidia of the isolates were gently scraped from the surface of two weeks old cultures and suspended into a tube containing distilled water and 0.02% Tween 80® solution (MERCK, Germany). The suspension was stirred and filtered through a single layer of linen to remove culture debris and mycelia. Conidial concentrations were estimated using a haemocytometer with a light microscope. The concentration in the primary solution was adjusted to 1×10^7 spores/ml by dilution with distilled water and Tween 80® and then used in experiments. Viability of the spores for both isolates was also

determined on SDA plates before being used in the experiments. Spores with a viability of more than 90% were used for the bioassay.

7.2.4 Treatments used in the field and greenhouse experiments

The following treatments were used in field and greenhouse experiments for determination of efficacy of three formulations of *M. anisopliae* isolate ICIPE 51 against *Rhopalosiphum padi* and *Metopolophium dirhodum*.

1. *R. padi/ M. dirhodum* + Water (Negative Control) = T1/ T6
2. *R. padi/ M. dirhodum* + ICIPE 51 + Water = T2/ T7
3. *R. padi/ M. dirhodum* + ICIPE 51 + Elianto Vegetable oil = T3/ T8
4. *R. padi/ M. dirhodum* + ICIPE 51 + Agronite (Sticker) = T4/ T9
5. *R. padi/ M. dirhodum* + Karate (Aphicide) + Water = T5/ T10

7.2.5 Effect of different formulations of *M. anisopliae* isolate ICIPE 51 on *R. padi* and *M. dirhodum* under field conditions.

The field trial was planted in a randomized complete block in a split plot arrangement and replicated three times and was repeated twice. The main plots were caged with a white netting material to prevent cross-contamination of aphid species. Over the two trials the locations remained constant but randomization was changed each year. The main plots which measured 12.5 x 5 m and separated by 1 m path were randomly allocated to aphid species. Formulations were randomly allocated to the sub-plots measuring 2 x 1 m and separated by 0.5 m paths. Each subplot had 8 rows spaced 0.2 m apart. The seeds were manually drilled in hill plots at a seed-rate of 125kg/ha in well ploughed and harrowed fine tilth. Di-ammonium phosphate (DAP) 18:46:0 (NPK) fertilizer was applied at the rate of 60 kg ha^{-1} at planting.

At two to three leaf stage (Zadok's growth stage 12-13) (Zadok *et al*., 1974) untreated plots were infested with seven to ten (5-7 day old) aphids per plant using a paint brush so as to boost aphid build up and multiplication. The aphids were allowed to establish on the plants for one week before being sprayed twice at 15-day interval with *Metarhizium anisopliae* (Metch) isolate ICIPE 51 at a concentration of 10^7conidia/ml using a hand sprayer. The fungus was applied in 3 formulations- sterile distilled water containing 0.05% Triton X-100,

oil-based formulation (Elianto vegetable oil) and sticker (Agronite). Negative control plots were treated with sterile distilled water containing 0.05% Triton X-100 while positive control plots were treated with a recommended insecticide (Karate) which has been widely applied for control of insect and aphid pests in Kenya. A cardboard was used to protect adjacent plots/treatments from drift of spray. Areas of 2 m-wide at the field edge and 1-m-wide between the plots were not sprayed as buffer.

Data was collected from the two middle rows. Initial counts of nymphs and adults of the aphids on wheat plants were made in situ the day before the first spray using a hand lens magnifier. After the first spray, aphid densities (number of aphids per plant) were monitored at 5-day intervals for 35 days. The efficacies of the different fungal treatments were evaluated based on the in situ counts of living aphids. Data on temperatures, humidity, and rainfall were collected over the test period (Appendix 2).

7.2.6 Effect of *M. anisopliae* isolate ICIPE 51 formulations on the fecundity of *R. padi* and *M. dirhodum* under greenhouse conditions.

Four replicates of five treatments (three fungal formulations and one positive and one negative control) were prepared as described in section 7.2.3 and inoculated to aphid groups each containing about 40 apterous adult aphids. This total included extra aphids to ensure that each treatment would have 30 live aphids after being treated with conidial suspension. The protocol of handling the control groups remained as described in section 7.2.5. Treated aphids were transferred to a leaf in an assay cell, one aphid per cell. Each assay cell consisted of a 60-mm transparent plastic Petri dish containing a 5-cm length of wheat leaf from a greenhouse-grown plant (2-3 wk old) with the ends contacting bands of water-soaked, sterile cotton. The assay cells were maintained in the ventilated Plexiglas cages. The cotton wool in the Petri dishes was saturated daily with water and every 3 - 5 days aphids were transferred to new leaf disks. New born nymphs were removed after counting using a hand lens. The treated aphids were observed daily for 7 days to record mortality and fecundity.

7.2.7 Comparison of pathogenic potential of the entomopathogenic formulations of *M. anisopliae* isolate ICIPE 51 when applied before (Pre) and after (Post) infestation of wheat with *R. padi* and *M. dirhodum* under greenhouse conditions.

The greenhouse trial for potted wheat plants was laid in a randomized complete block in a split- split plot design and replicated three times and repeated twice. Over the two trials the locations remained constant but randomization was changed each year. Aphid species and mode of infestation were randomly allocated to the main plots and subplots respectively. Formulations were randomly allocated to the sub-plots. At two to three leaf stage (Zadok's growth stage 12-13) (Zadok *et al*., 1974) half of the potted wheat (Post-infestation subplots) were artificially infested with seven to ten (5-7 day old) aphids obtained from a colony maintained at the greenhouse and allowed to establish in the containers for 7–10 days prior to fungal treatment with three formulations and treatment with negative and positive controls as outlined in section 7.2.5. The remaining half (Pre-infestation subplots) were treated with the fungus and controls prior to infestation with the two aphid species. Individual containers were covered by cotton netting material to allow good aeration but prevent escape of aphids.

Initial counts of nymphs and adults of aphids were made in situ the day before the first spray using a hand lens magnifier. After the first spray, aphid densities were monitored at 5-day intervals for 15 days.

7.3 Data Analysis

Variances of conidial deposits, aphid densities, percent density declines and relative efficacies among the treatments and the sample occasions were analyzed using a two-way procedure for analysis of variance (ANOVA) in SAS software (SAS, 2003). The percent decline of aphid density (D) and the efficacy (E) of each treatment relative to the negative control were computed as $D = (d_{T0} - d_{Ti})/d_{T0} \times 100$ and $E = (d_{C0}d_{Ti})/(d_{Ci}d_{T0}) \times 100$, where d_{T0} and d_{Ti} were mean aphid densities (no. aphids per plant) estimated from a given treatment on the initial sample day and the specific sampling day after the first spray; d_{C0} and d_{Ci} were the densities estimated from the negative control on the initial sample day and the specific sampling day after the first spray. The levels of aphid control by the different fungal

formulations were compared based on the estimates of both indices. The data was analyzed using a one-way analysis of variance (ANOVA); means were separated by LSD at 0.05 level.

7.4 Results

7.4.1 Trends of aphid densities and field efficacies of fungal formulations

The results showed that fungal spores formulated with either water, oil or sticker had pathogenic effects on both *R. padi* and *M. dirhodum*. Comparison of formulations showed a significant ($F(79,160) = 37.04$, $P < 0.05$) decline in aphid densities when spores of the fungi were applied in either oil or sticker as opposed to water (Figure 7.1). Initial mean densities of the living nymphs and adults of aphids, in all the treatments were averaged as 12.8 ± 2.5 per plant. Aphid densities were greatly reduced by the three fungal formulations on days 5 and 10 after the first spray (Figure 7.1) and rebounded slightly on day 15. The second spray of fungus for oil and sticker formulations on day 16 further suppressed aphid densities to lower levels for another 20 days. In contrast, maximal aphid densities were consistently observed from the plots of negative control throughout the trials, followed by fungus in water formulation. The plots with positive control formulation of Karate aphicide registered drastic reduction of aphid densities followed by immediate consistent increase after 10 days. The two-way ANOVA of aphid densities showed significant differences among the treatments and sample days ($P < 0.05$).

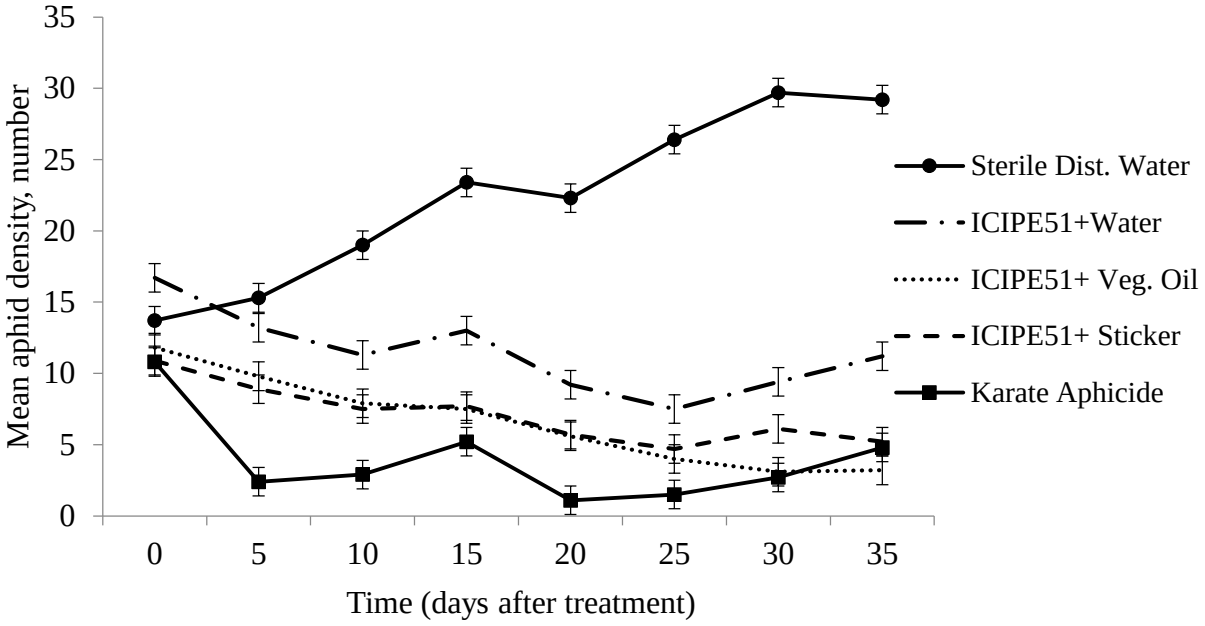

Figure 7.1 Effect of different formulations of *M. anisopliae* isolate ICIPE 51 on mean aphid density over days after two sprays. The second spray was provided on day 16. Failure of error bars to overlap was used as the criteria for identifying significant differences among means of tested formulations

Based on counts of living aphids on each of the sampling occasions, the percentages of density declines and relative efficacies in all the treatments of both trials were computed. Both indices differed significantly ($P < 0.05$) among the treatments or sample days. Some estimates of the indices from the treatments of the negative controls were negative, denoting that aphid populations had actually increased over time. All the fungal candidates provided significant controls of the aphids for 35 days when sprayed twice at 15-day interval (Figure 7.2). The aphid densities were significantly suppressed by the first spray and further reduced to lower levels by the second spray despite some differences among the fungal formulations.

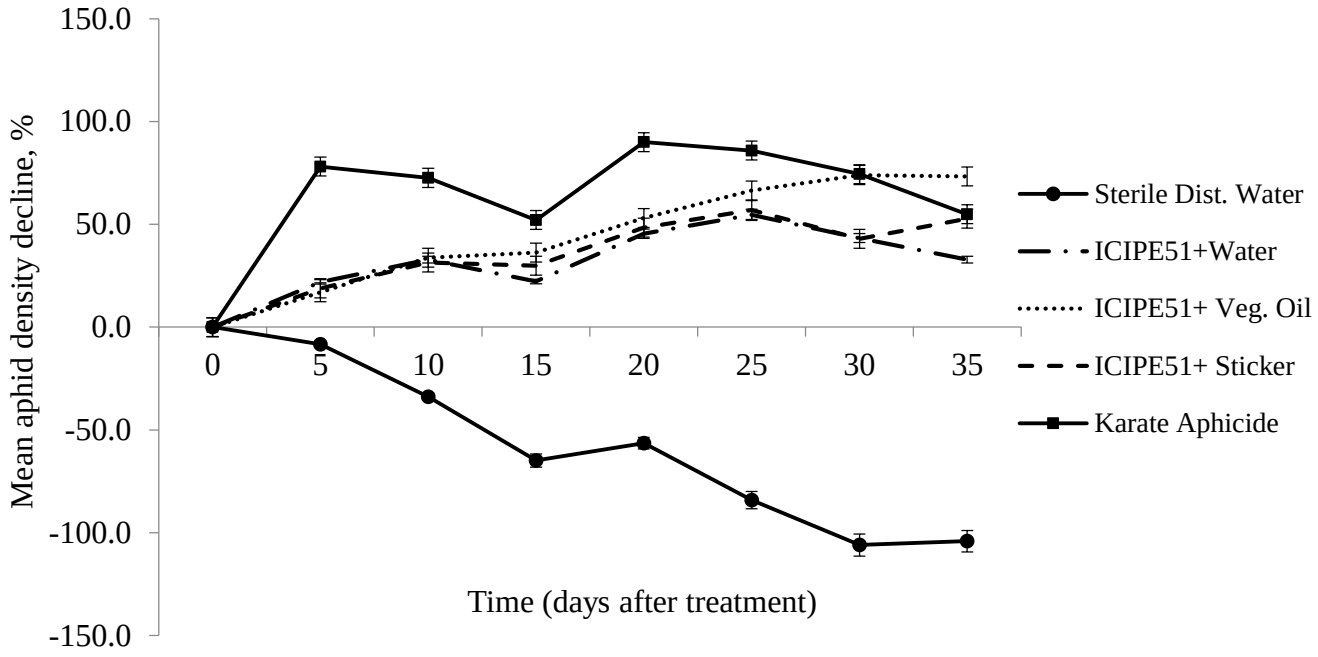

Figure 7.2 Effect of different formulations of *M. anisopliae* isolate ICIPE 51 on mean aphid density decline over days after two sprays. The second spray was provided on day 16. Failure of error bars to overlap was used as the criteria for identifying significant differences among means of tested formulations

Based on aphid density declines and relative efficacies, vegetable oil formulation was best among the tested candidates, followed by the sticker formulation (Figure 7.3). Overall means of aphid density decline from the sampling occasions of day 15 and day 35 were 36.2% and 73.3% for fungus with vegetable oil, 29.9% and 52.8% for fungus with sticker and 22.2% and 32.8% for aqueous fungal formulation respectively. These corresponded to overall mean relative efficacies on day 35 of 13.5%, 24.5% and 35.4% respectively. The best control was always observed 10 or 15 days after the second spray regardless of the fungal candidates. Results showed that aphid density decline and percent relative efficacy of vegetable oil fungal treatments were significantly greater than indices from negative control and positive Karate aphicide control. The negative means of density declines in the blank controls and water-based fungal formulation indicate virtual increases of the aphid populations.

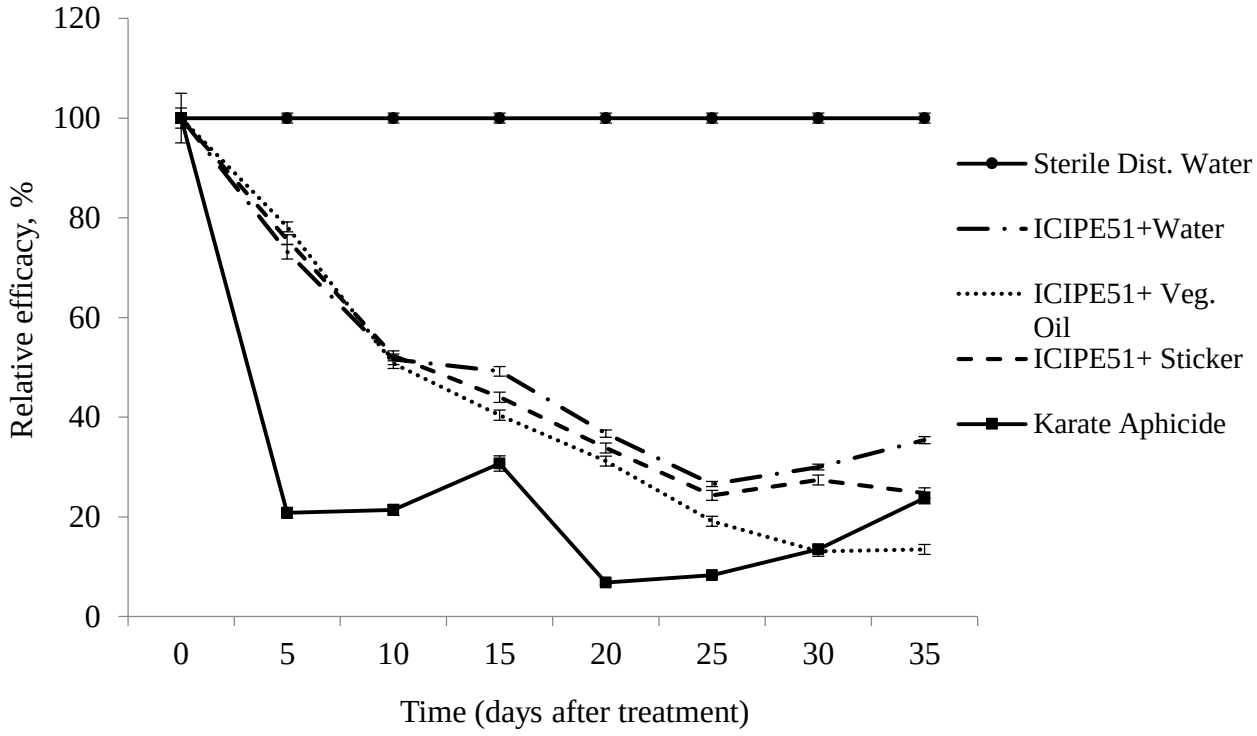

Figure 7.3 Relative efficacies of different formulations of *M. anisopliae* isolate ICIPE 51 over days after two sprays on aphids. The second spray was provided on day 16. Failure of error bars to overlap was used as the criteria for identifying significant differences among means of tested formulations

Whereas the results indicated that aphid species by days after spraying interaction was not statistically significant ($p= 0.0576$ and 0.0628 for aphid density and mean density decline respectively), treatments (formulation) showed significant interaction ($p< 0.05$). *M. dirhodum* was significantly more susceptible to control by the three formulations than *R. padi*. (Table 7.1). Thus, the fungus with vegetable oil showed 48.6% and 38.0% relative efficacy against *M. dirhodum* and *R. padi* respectively. Similarly, when the fungus was formulated with sticker relative efficacy against *M. dirhodum* and *R. padi* was 50.9% and 44.6% respectively.

Table 7.1: Effect of field control of *M. dirhodum* and *R. padi* by different formulations of *M. anisopliae* isolate ICIPE 51 on mean aphid density, percentage density decline and relative treatment efficacy

Treatment	Mean aphid Density		Mean Density Decline (%)		% Relative Efficacy	
	M. dirhodum	*R. padi*	*M. dirhodum*	*R. padi*	*M. dirhodum*	*R. padi*
Sterile Distilled Water	13.9	30.8	5.1	4.3	100.0	100.0
ICIPE51+Water	9.2	13.6	5.4	5.4	57.0	43.6
ICIPE51+ Veg. Oil	6.4	6.8	5.5	5.5	48.6	38.0
ICIPE51+ Sticker	6.4	7.8	5.4	5.4	50.9	44.6
Karate	4.2	3.7	5.5	5.5	32.4	23.8
LSD	1.0		0.2		2.0	
CV (%)	23.6		5.7		9.1	

7.4.2 Effect of formulations on aphid fecundity and intrinsic rate of increase.

Comparison of the fungal formulations effect on population attributes showed that the three formulations were more effective against *M. dirhodum* than *R. padi*. There was significant difference among treatments over days after initial spray: cumulative fecundity ($F = 44.17$; $df = 69,490$; $p< 0.05$) and intrinsic rate of increase, r_m ($F = 39.05$; $df = 69,490$; $p< 0.05$). ICIPE 51 isolate formulated in sticker and the fungus formulation in vegetable oil were significantly less fecund than the other treatments. The aqueous fungal formulation registered the highest average fecundity and intrinsic rate of increase of 1.5 nymphs per aphid and 0.42 nymphs/aphid/day. This was comparable to fecundity 1.4 nymphs/aphid recorded among the positive aphicide controls. Fungal formulations formulated in either oil or sticker showed the slowest average rate of intrinsic increase of 0.32 and 0.31 respectively as compared to water formulation and positive aphicide controls which registered indices of 0.42 and 0.38 respectively (Figure 7.4 and Figure 7.5).

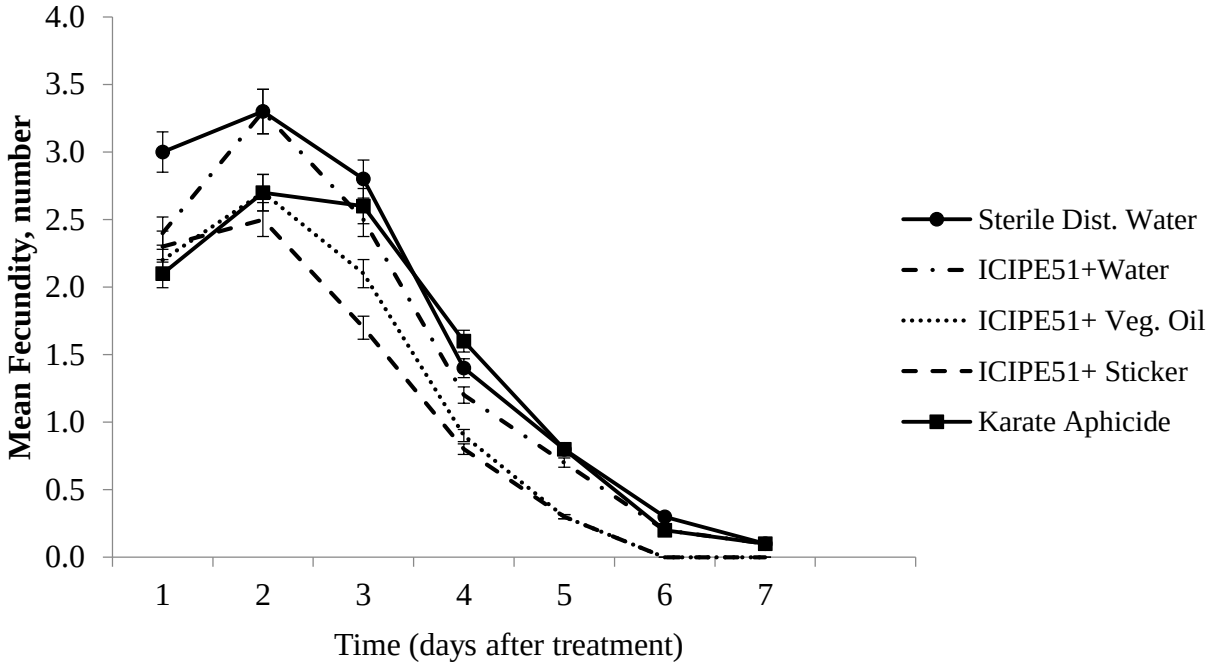

Figure 7.4 Effect of different formulations of *M. anisopliae* isolate ICIPE 51 on fecundity of aphids over 7 days after initial spray on greenhouse grown wheat

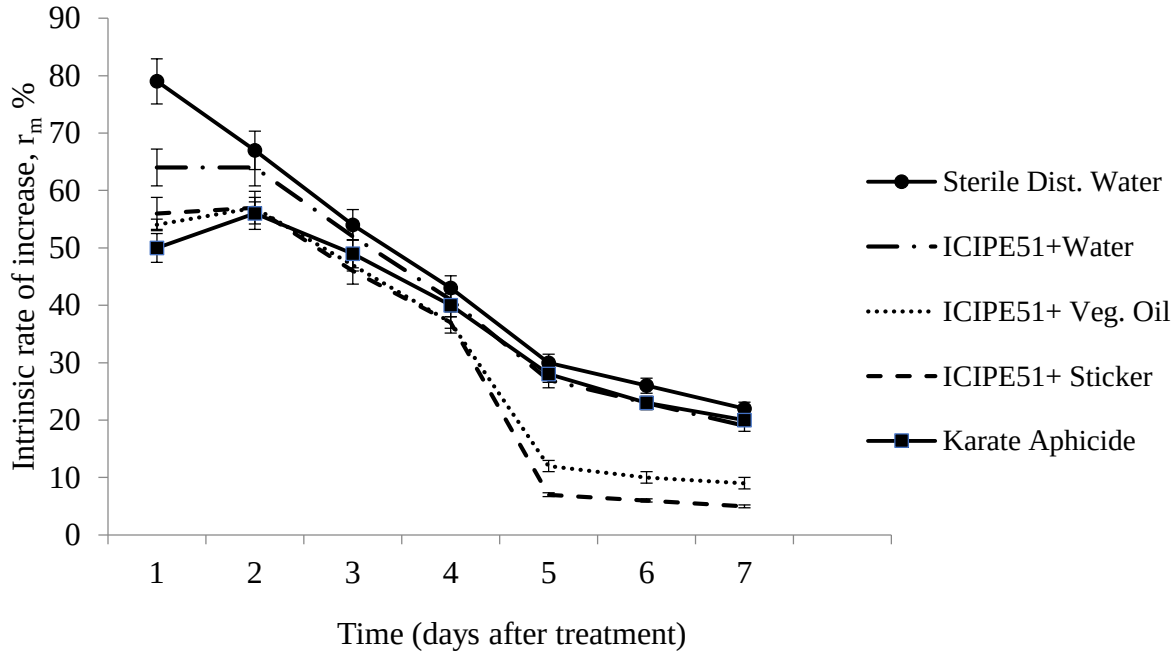

Figure 7.5 Effect of different formulations of *M. anisopliae* isolate ICIPE 51 on intrinsic rate of increase of aphids over 7 days after initial spray on greenhouse grown wheat

7.4.3 Pathogenic potential of modes of fungal formulations application.

Results showed that the fungal formulations applied either before or after aphid infestation of wheat plants had pathogenic effects on the aphids. Comparison of aphid density decline and relative efficacy indices indicated significant differences between modes of fungal formulation application: Aphid density ($F(79,400) = 10.25$, $P < 0.05$) and percent relative efficacy ($F(79,400) = 43.61$, $P < 0.05$). The interaction of mode of application with either aphid species or days after initial spray was not statistically significant ($p = 0.463$ and 0.199 respectively).

Post infestation application of *M. anisopliae* isolate ICIPE 51 induced greater and significant ($p<0.05$) decline in aphid densities and percent relative efficacy than pre infestation application regardless of the formulation (Table 7.2).

Table 7.2: Effect of mode of application of different *M. anisopliae* isolate ICIPE 51 formulations against aphids on mean aphid density, percentage density decline and relative treatment efficacy on greenhouse grown wheat

Treatment	Mean aphid density (%)		Mean density decline (%)		% Relative efficacy	
	Post	Pre	Post	Pre	Post	Pre
Sterile Distilled Water	19.7	22.8	-25.5	-74.0	100.0	100.0
ICIPE51+Water	15.4	14.7	9.8	-13.8	76.0	71.4
ICIPE51+ Veg. Oil	14.1	10.0	30.3	16.2	64.1	57.7
ICIPE51+ Sticker	12.7	9.5	26.5	22.4	66.6	55.2
Karate	11.8	13.3	38.9	-13.5	51.3	71.7
LSD	1.3		4.9		2.9	
CV (%)			28.1			

Pre-infestation and post-infestation mean aphid density decline for the aqueous formulation was -13.8% and 9.8%, 16.2% and 30.3% respectively for the oil based formulation and

22.4% and 26.5% respectively for the fungal spores formulated in sticker. Likewise, pre-infestation and post-infestation percent relative efficacy for fungus in water formulation was 71.4% and 76.0% respectively and 57.7% and 64.1% for the oil based formulation and 55.2% and 66.6% respectively for the fungal spores formulated in sticker. Post infestation percent relative efficacy of all three tested formulations was significantly higher than 51.3% of the positive Karate aphicide control.

For pre-infestation application, spores formulated in sticker significantly outperformed the oil-based formulation. Sticker based formulation induced a 22.4% aphid density decline as compared to 16.2% reduction for the oil based formulation. The aqueous formulation just like the positive aphicide and negative blank control registered negative means of the density declines indicating virtual increases of aphid populations.

7.5 Discussion

All the three *M. anisopliae* ICIPE 51 formulations tested were more effective in reduction of population densities of *M. dirhodum* and *R. padi* in the greenhouse and field trials as compared to the untreated controls. However, there was considerable variation between them. The study identified the oil based formulation as the most superior, followed closely by the sticker based formulation. Aqueous conidia formulation was least effective. Many studies have reported variations in pathogenic activity and efficacy of fungal formulations on arthropod pests (Kaaya and Hassan, 2000; Batta, 2003; Wekesa *et. al*., 2005). This underlines the importance of proper selection of formulation.

Enhanced infectivity has been reported when conidia of *M. anisopliae* (Prior *et al*., 1988) were formulated in oil rather than water. This increased infectivity could be a result of wider spread and stronger adhesion of the oil to the cuticle, possibly reaching intersegmental membranes, in contrast to the water formulation. Conidia of *B. bassiana* isolate GPK and *M. anisopliae* isolate ICIPE 78 formulated as emulsifiable oil caused higher *T. evansi* mortality than the ones formulated in water. The enhanced infectivity of conidia formulated in oil formulations was first reported by Prior *et al*. (1988). Kaaya and Hassan (2000) found that

oil-based formulations of conidia of B. bassiana and *M. anisopliae* were more effective against *Ripicephalus appendiculatus* Neumannn than the aqueous formulation. Batta (2003) also reported that conidia of *M. anisopliae* formulated in an invert formulation (water-in-oil formulation) with a coconut/soybean oil preparation were more effective against *T. urticae* than nonformulated conidia. Although aqueous formulation of Silwet L-77 has been shown to be most toxic to the two spotted spider mite, *T. urticae*, and the Pacific spider mite, *T. pacificus* McGregor (Cowles *et al*. 2000; Tipping *et al*. 2003), this was not observed in our study. Greater efficacy of entomopathogenic fungi spores formulated in oil has also been demonstrated against acridid grasshoppers. Inglis *et al*. (1996) found that the formulation of *B. bassiana* (10^5 conidia) spores in oil was more effective (95% mortality) against the grasshopper *Melanoplus sanguinipes* (Fabricius) than those in water (55% mortality). The present study found similar results. The rate of mycosis and mortality found in the present study differs naturally from other studies because of the differences in fungal isolates, target insect and spore concentrations used in the studies.

The reduced fecundity and slow rate of intrinsic increase of *R. padi and M. dirhodum* observed in aphids treated with oil based formulation may be attributed to increased infectivity leading to induction of higher reduction in feeding as compared to other formulations. This may affect aphid body fat accumulation at sexual maturity and consequently reproductive potential of infected, but surviving insects. Effect of infection *M. anisopliae* on the oviposition and reproductive potential of various insect hosts has been investigated in some studies (Arthurs and Thomas, 2000; Migiro *et al*., 2011; Ondiaka *et al*., 2008).

The reasons why pre-infestation fungal application registered minimal reductions in aphid densities as compared to post infestation application may be attributed to epicuticular wax or bloom that occurs in common bread wheat consisting mainly of straight-chain aliphatic hydrocarbons serving to decrease surface wetting and moisture loss. According to Narhe and Beysens (2006) and Holloway and Jeffree (2005), epicuticular wax forms crystalline projections from the plant surface, which enhance their water repellency, creating a self-

cleaning property known as the lotus effect (Narhe and Beysens, 2006 and Holloway and Jeffree, 2005).

In conclusion, our greenhouse and field trial results indicated that *M. anisopliae* isolate ICIPE 51 has a great potential for management of *R. padi and M. dirhodum*, particularly when formulated in oil. However, if the entomopathogen is to be applied prophylactically, before infestation of wheat with aphids, then a sticker-based formulation would be most preferable. The positive effects of fungal/chemical interactions on field control have been confirmed on field control of whiteflies (Feng *et al*., 2004a), tea leafhoppers (Feng *et al*., 2004b) and citrus red mites (Shi and Feng, 2006). Thus, more field studies are needed to demonstrate the effect of the fungal/chemical interaction on the control of the *R. padi* and *M. dirhodum* in Kenya. Further, additional experiments are required to determine the effects of wheat cultivars on the viability and infectivity of the fungus since certain proteins such as cysteine-rich defensins, widely distributed in wheat leaves display broad anti-microbial activity and may cause varying degrees of inhibition of colony formation and growth of entomopathogenic fungi (Freeman and Beattie, 2008). Overall, apart from the technical aspects and climate conditions, the use of the formulation must consider costs associated with spore production and application.

CHAPTER EIGHT
CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

The success of entomopathogenic fungi as microbial control agents requires careful and appropriate selection of the most efficacious species and isolates. In this study, the pathogenicity of six isolates of *Beauveria bassiana* (Bals.) Vuill. and 14 isolates of *Metarhizium anisopliae* (Metsch.) Sorok against *Metopolophium dirhodum (Walker) and Rhopalosiphum padi* (L) was for the first time determined in the laboratory. The results of time-mortality (LT_{50}) laboratory study showed that three out of the twenty screened isolates, identifiable as ICIPE 84, ICIPE 51 and ICIPE 23 registered the shortest periods of lethal infection, indicating high level of pathogenicity or virulence of the select isolates.

The selected isolates were further subjected to dose-mortality bioassays in an effort to critically evaluate their bioefficacy against *R. padi* and *M. dirhodum*. Dose-response results indicated that ICIPE 51 outperformed isolates ICIPE 23 and ICIPE 84 by recording the lowest LC_{50} and LC_{90} values. Virulence has always been one of the most important parameters considered for strain selection (Inglis et al. 2001). The difference in the LC_{50} values of strains belonging to the same fungal species could be attributed to differential virulence and to genetic variations arising from specialization toward a determined host and the geographical distribution of the strains (Coates *et al*., 2002).

When considering the effectiveness of an entomopathogen against a host with a high reproductive potential, estimation of adult mortality alone may not be satisfactory. This caution should be extended to advice against testing pathogenicity against only one developmental stage. No studies had been undertaken, hitherto, to investigate the differential susceptibility of different developmental stages of *Rhopalosiphum padi* (L.) and *Metopolophium dirhodum* (Walker) to fungal infection. Virulence for all stages was dose-dependent and mortality increased with host aphids' maturity and over time. These results showed that *M. anisopliae* isolate ICIPE 51 could be a viable alternative to chemical control of *R. padi* and *M. dirhodum* in bread wheat.

Arthropod fecundity and fertility are affected by many factors including pathogens. It has been demonstrated that entomopathogenic fungi may cause sublethal reproductive effects on target individuals that can have important implications for the population dynamics of the host and contribute to the status of the target insect as a pest. Results indicated, for the first time, the pathogenicity of *M. Anisopliae* isolate ICIPE 51 against *R. padi* and *M. dirhodum* under controlled laboratory conditions. Virulence was dose-dependent and mortality increased with time. Low doses of the isolate did not affect pre-lethal reproductive effects, such as fecundity and intrinsic rate of increase. Both aphid species were significantly more fecund in their early adulthood, suggesting the stage as ideal for biopesticide management intervention. The reduction of the reproductive potential of *M. dirhodum* and *R. padi* adults that are fungally challenged may contribute to the overall efficacy of the treatment.

Successful use of entomopathogenic fungi as microbial control agents ultimately depends on the use of the right propagule, formulated in an optimum fashion and applied at the right time to a susceptible host. The formulation of entomopathogenic fungi against *R. padi* and *M. dirhodum* had not been studied previously in Kenya. Greenhouse and field trials established that *M. anisopliae* isolate ICIPE 51 has a great potential for management of *R. padi and M. dirhodum*, particularly when formulated in oil. However, if the entomopathogen is to be applied prophylactically, before infestation of wheat with aphids, then a sticker-based formulation would be most preferable.

8.2 Recommendations

Recommendations for future work and next steps for continuation of this study should include:

1. Further additional experiments to determine the effects of wheat cultivars on the viability and infectivity of the fungus since certain proteins such as cysteine-rich defensins, widely distributed in wheat leaves display broad anti-microbial activity and may cause varying degrees of inhibition of colony formation and growth of entomopathogenic fungi (Freeman and Beattie, 2008).

2. Finding more virulent fungal isolates: Research should be continued to identify more virulent strains of the fungi. Several methods are available for collecting insect pathogens. To obtain virulent isolates the following steps should be followed:

i) Screening to determine the most virulent isolates among the Kenyan culture collections (ii) screening to determine the most virulent isolates among exotic isolates (iii) obtaining new strains from *R. padi* and *M. dirhodum* or other aphid hosts.

3. Studying characteristics of virulent isolates: Apart from virulence other characteristics of selected isolates such as conidial viability, storage qualities, and environmental tolerance need to be assessed. Therefore, continued expansion of research on fungal characteristics is needed so that the factors limiting mycosis development under field conditions will be better understood.

4. Further field assessment: The pathogenicity of the fungi selected via screening should be further assessed in field conditions to ascertain their efficacy against aphids in field conditions.

5. Mass production, formulation, application and long term preservation: The selected entomopathogenic fungi isolates will need to be preserved for a long time; however their virulence and characteristics may be affected by the media during preservation. Further studies need to be done in order to find the best artificial media and conditions for long-term preservation of the fungi without losing virulence. Research is also needed to find the best way for mass production of the fungal strains. Overall, apart from the technical aspects and climate conditions, the use of the formulation must consider costs associated with spore production, and application.

6. The use of selected fungal isolates as a component of Integrated Pest Management (IPM) programmes.

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APPENDICES

Appendix 1: Growth stages of wheat code based on Zadok *et al*., 1974

Code	Zadok's Stage visual description	Code	Zadok's Stage visual description
0	Germination		
00	Dry seed	4	Booting
01-09	Start of imbibition to leaf just at coleoptile tip	41	Flag leaf sheath expanding
		43	Boots just visible
1	Seedling growth	45	Boots swollen
10	First leaf through coleoptiles	47	Flag leaf opening. Flag leaf sheath Opening
11	First leaf unfolded	49	First awns visible
12	2 leaves unfolded		
13	3 leaves unfolded	5	Ear emergence
14	4 leaves unfolded	51	First spikelet of ear just visible
15	5 leaves unfolded	53	One-fourth of ear emerged
16	6 leaves unfolded	55	One-half of ear emerged
17	7 leaves unfolded	57	Three fourths of ear emerged
18	8 leaves unfolded	59	Emergence of ear completed
19	9 or more leaves unfolded		
		6	Flowering
2	Tillering	61	Beginning of flowering
20	Main shoot only	65	Flowering halfway complete
21	Main shoot and 1 tiller	69	Flowering complete
22	Main shoot and 2 tiller		
23	Main shoot and 3 tiller	7	Milk development
24	Main shoot and 4 tiller	71	Seed water ripe
25	Main shoot and 5 tiller	73	Early milk
26	Main shoot and 6 tiller	75	Medium milk, contents mostly milky liquid

27	Main shoot and 7 tiller	77	Late milk, liquid endosperm when crushing seed
28	Main shoot and 8 tiller		
29	Main shoot and 9 tiller	8	Dough development
		83	Early dough
3	Stem elongation	85	Soft dough (fingerprint impression not held)
30	Pseudostem erection	87	Hard dough (fingerprint impression held, head loosing chlorophyll)
31	1st node detectable		
32	2nd node detectable	9	Ripening
33	3rd node detectable	91	Seed hard (difficult to divide by thumbnail)
34	4th node detectable	93	Seed loosening in daytime
35	5th node detectable	95	Seed dormant
36	6th node detectable	96	Viable seed giving 50% germination
37	Flag leaf just visible	97	Seed not dormant
39	Flag leaf ligule just visible	98	Secondary dormancy induced
		99	Secondary dormancy lost

Appendix 2: Rainfall and temperatures prevailing over the experiment period at Njoro

Year	January	February	March	April	May	June	July	August	September	October	November	December
2011												
Rainfall (mm)	3.9	9.5	130.3	28.9	120.5	177.7	158.6	124.9	145.4	103.1	165.3	104.6
Temp (Min)	8	8	9	9	9	11	11	12	11	11	11	15
Temp (Max)	25	26	26	25	23	21	18	15	22	22	20	16
2012												
Rainfall (mm)	0	13.6	11	0	0	0	87.3	174.7	174.9	0	0	112.7
Temp (Min)	10	16	18	14	12	12	10	10	9	11	14	13
Temp (Max)	23	18	22	24	22	22	20	20	22	23	21	21

Appendix 3: List of publications

1. **Murerwa, P**., Arama, P. F., Kamau, A. W. and Maniania, N. K. (2014a). Pathogenicity of Fungal Isolates of *Metarhizium anisopliae* (Metchnikoff) and *Beauveria bassiana* (Balsamo) against Aphids *Rhopalosiphum padi* (Linnaeus) and *Metopolophium dirhodum* (Walker). *Egerton Journal of Science and Technology*, 14: 105-119.

2. **Murerwa, P**., Arama, P. F., Kamau, A. W. and Maniania, N. K. (2014b). Effect of Infection by *Metarhizium anisopliae* isolate ICIPE 51 on developmental stage, fecundity and intrinsic rate of increase of *Rhopalosiphum padi* and *Metopolophium dirhodum. Journal of Entomology and Nematology*, 6(11): 154-160. DOI:10.5897/JEN2014.0114