ENTOMOPATHOGENICITY OF HYPHOMYCETE FUNGI TO FRUIT FLY *Bactrocera invadens* (Diptera: Tephritidae) AND THEIR POTENTIAL FOR BIOLOGICAL CONTROL ON MANGO

BY

OUNA ELIZABETH AWUOR

REG. NO. I56/11332/2004

"A THESIS RESEARCH SUBMITTED IN PARTIAL FULFILMENT OF THE DEGREE OF MASTER OF SCIENCE (MICROBIOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA"

FEBRUARY 2010

DECLARATION BY CANDIDATE

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

Signature_____ Date_____

Ouna Elizabeth Awuor

DECLARATION BY SUPERVISORS

This thesis has been submitted with our approval as University and ICIPE supervisors

Signature_____ Date _____

Dr. Jonah Birgen

Department of Plant and Microbial Sciences

Kenyatta University

Signature_____ Date _____

Dr. Sunday Ekesi

African Fruit Fly Initiative

International Center of Insect Physiology and Ecology

DEDICATION

To my mother, *Jane Margaret Wanunda,* father *Zebedi Ouna Obare* and my late aunt Sylvia Akello for all the love and support.

ACKNOWLEDGEMENT

I am especially indebted in many ways to the following persons and donor whose contribution made this dissertation possible.

Thank you Dr. Ekesi for your initiative to provide me with research project, vital supervision and leadership of the African Fruit Fly program. Without doubt you were the key to making this thesis to what it is.

Thank you Dr. Birgen for the dedication and criticism of this dissertation and for taking me through several scientific administrative steps from the twisted beginning when I lost my initial supervisor.

Thank you Dr. N. K. Maniania for provision of time to attend course work and for providing me with the facility to carry out my research work within the Arthropod Pathology laboratories of ICIPE.

Many thanks to Capacity Building Unit of ICIPE for the training opportunity granted through ICIPE Staff Development Program.

Thank you lectures in the Department of Plant and Microbial Science of Kenyatta University for the academic knowledge offered in class and during seminar presentations.

Many thanks to BMZ, Germany and RUFORUM for funding this project, sponsorship for training in Scientific Data Management and in fruit fly taxonomy.

Thank you Mr. John Kilu for your constant supply of experimental fruit flies used throughout my research period.

Thank you Brenda and Oliver for encouraging me to pursue this study.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

PAGE

LIST OF PLATES

PAGE

ACRONYMS AND ABBREVIATIONS

AFFI	Africa Fruit Fly Initiative
ANOVA	Analysis of Variance
BCA	Biological Control Agent
CI	Chemical insecticides
DBM	Diamondback moth
DDT	Dichlorodiphenyltrichloroethane
EPF	Entomopathogenic Fungus
EU	European Union
FAO	Food and Agriculture Organization
GPS	Geographical position point
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
IITA	International Institute of Tropical Agriculture
LUBILOSA	Lutte Biologique Contre les Locusts et Sauteriaux
MRL	Maximum Residue Level
ME	Methyl Euginol
MI	Microbial insecticide
MCA	Microbiological control Agent
PHI	Pre-harvest Interval
SDA	Sabouraude Dextrose Agar
SNK	Student-Newman Keuls’
WHO	World Health Organization

ABSTRACT

Sustainable mango production will rely increasingly on alternatives to conventional chemical insecticides that are environmentally friendly for the management of fruit flies. The use of microbial control agents such as fungi in pest suppression is considered suitable since micro-organisms usually exert low environmental impact and are target specific. Application methods of bio-pesticides in the environment, which use minimal amount of inoculum is currently under improvement from inundative to auto-inoculative methods. Such devices are usually designed to attract insects into focus of the entomopathogen and use the insect as a vector for transmission of pathogen to other members of its population. Virulence of 24 isolates of *Metarhizium anisopliae* (Metschnik.) Sorok. and *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocrales) collected from the microbial culture collection at International Center of Insect Physiology and Ecology and isolates collected pathogen survey of the study was evaluated on immature adult *Bactrocera invadens* Drews, Tsuruta and White in the laboratory, followed by testing sub-lethal effects of one of the most pathogenic isolates on fecundity and fertility. Out of the 24 isolates of *M. anisopliae* and *B. bassiana* screened for pathogenicity against *B. invadens*, 7 isolates (ICIPE 20, 43, 62, 69, 295, 303 and *M. anisopliae var. acridium* IMI 330189 or ICIPE 21, available as Green Muscle® induced significantly higher mortalities (ranging from 93.7% to 94.8%) than the rest of the isolates. Lethal time to 50% mortality (LT_{50}) ranged from 2.8 to 3.6 days (*M. anisopliae*) and 2.7 to 2.8 days (*B. bassiana*); and slope value estimates of 0.96 and 0.88 respectively at 5 days post treatment. Sub-lethal effect of selected isolate of *M. anisopliae* (ICIPE 20) on *B. invadens* exhibited significantly lower egg fecundity and hatchability in treated flies than control at levels of 6.8 and 28.4 eggs per fly per day and a gross of 28.8 eggs against 113.2 eggs/female at 4 days post inoculation; and hatchability of 17.2% and 64.8% respectively. Evaluation of three different bait formulations contaminated with *M. anisopliae* was carried out based on resident time flies spent on the baits, persistence of conidia on the baits exposed in the field under mango canopy and pathogenicity of fungus contaminated baits to adult *B. invadens* in the laboratory at 0, 7, 14, 21, and 28 days of exposure in the field under mango canopy.This caused an overall reduction in fecundity between 80.2 - 99.8% and 51- 80% in hatchability. Resident time spent by *B. invadens* on *M. anisopilae*-contaminated food baits was not significantly different among the 3 baits (2% NuLure, local bait DuduLure® and a diluted 10% DuduLure); however germination of conidia from 10% DuduLure and 2% NuLure was significantly higher than that DuduLure® at 14, 21 and 28 days of field exposure and were at the levels of 83%, 96%, 78% (10% DuduLure); 74%, 95%, 96% (2% NuLure); 54% 6%, 6% (DuduLure®). Two-way ANOVA demonstrated there was significant effect of time and environmental factors on conidial germination. Mortality of flies treated with fungus contaminated- 10% DuduLure and 2%NuLure baits was significantly higher than in treated DuduLure®. Mortality of flies exposed treated 10% DuduLure (84%) was significantly higher than 2% NuLure (64%) at 21 days but was significantly lower (48%) than 2% NuLure (90%) by 28 days. In conclusion this study has identified 7 new hyphomycete isolates as highly pathogenic to adult stages of *B. invadens*. Among the isolates *M. anisopliae* ICIPE 20 can cause reduction in egg fecundity and hatch rate. Wooden sphere soaked in commercial (NuLure) or locally developed bait (DuduLure®) and contaminated with spores of *M. anisopliae* can provide a viable option for the management of *B. invadens* on mango. The

adaptability of this technology will depend in part on its efficacy to suppress populations of *B. invadens* in the field.

CHAPTER 1

1.0 INTRODUCTION

Mango, *Mangifera indica* L., is an important fruit crop and key component of horticultural system in many tropical and subtropical parts of Africa. It is gaining recognition among tropical fruits as a dietary preference in the large urban and rural populations in Africa, serving as a source of vitamin A and other nutrients. Across the Sub-Saharan Africa, smallholder farmers who trade it as a source of cash income grow 90% of mango produce. Mango is the most internationally traded tropical fruit after pineapple in terms of volume and value (FAO IG-SG TF, 1999), and its export from Africa is estimated at 35,000 to 40,000 tons annually worth over US$42 million (Lux *et al*., 2003). Mango trade therefore sustains economies of many countries in Africa, serving as a source of revenue and jobs. For example, in South Africa, 55-60% of gross value of its agricultural export comes from fruit industry. In Kenya, a wide range of local, exotic and improved cultivars are grown (HCDA, 2002) and production is spread through diverse agro- ecological zones and altitudes, ranging from 0 to 1600 meters above sea level. Apple, Ngowe, Kent, Kett, Tommy Atkins and Haden are important cultivars for export market. Besides, in Kenya horticulture ranks highest export revenue earner with an estimated value of Kshs. 70 billion (US$ 14.7 million), fresh mango export produce is estimated at 40% with an estimated 197,000 tons produced in 2001 rising to 402,000 by 2007 (FAO, 1999; HCDA, 2004, 2007).

Mangoes, however, are attacked by a wide variety of pests and diseases, and fruit flies are regarded as one of the most important (Pena *et al*., 1998). Among the most important

fruit flies attacking mangoes are species of *Ceratitis cosyra* Walker, *C. fasciventris* Bezzi, *C. rosa* Karsh, *C. anonea* Graham, *C. capitata* Weidman and a recently introduced *Bactrocera* species of Asian origin, now described as *B. invadens* (Drew *et al*., 2005). In Kenya, estimates carried out by African Fruit fly Initiative program in 1999 showed that out of 90,000 tons of mangoes produced annually in the country, an estimated 20-40% of the fruit is lost to fruit fly infestation at the time of ripening. Among the commercial producers who grow it, losses due to fruit fly range from 20-25% and 30-80% at professional and smallholder levels respectively (Lux *et al.,* 2003). Producer countries may also lose potential markets due to stringent quarantine regulations imposed by importing countries to avoid entry and establishment of alien fruit flies.

Asian fruit fly pests from the genus *Bactrocera* are regarded as one of the most destructive insects of fruits and vegetables worldwide with adult traits that can include high dispersive powers, reproductive rate and extreme polyphagy. *Bactrocera* species are well-documented invaders and rank high on quarantine list world-wide (White and Elson-Harris, 1992; Clarke *et al*., 2005). *Bactrocera invadens*, believed to have invaded Africa from the Indian subcontinent, was discovered in Sri Lanka after it was first reported from Kenya (Lux *et al*., 2003) where it has become a significant pest of quarantine and economic importance. Prior to invasion by *B. invadens*, *C. cosyra* was the main mango pest in Kenya. Apart from mango the insect has over 30-host record, with mango being the most preferred host (Ekesi *et al*., 2006). The Inter-African Phytosanitary Council has described *Bactrocera invadens* as a "devastating quarantine pest" (French, 2005). The

pest is rapidly spreading across tropical Africa and in addition to Kenya it has spread to 22 African countries including Comoros Island (Drew *et al*., 2005; Ekesi *et al.,* 2006).

Management of mango infesting fruit flies in Kenya is largely dependent on use of chemical pesticides among commercial growers while the smallholder farmers largely depend on early harvest to evade damaging fruit fly populations. Both methods have considerable consequences because application of broad-spectrum pesticides leads to elimination of beneficial non-targets, development of pest resistance, resurgence of secondary pests and environmental pollution. *Bactrocera invadens* is among some of the *Bactrocera* species of fruit flies which can lay eggs on green mangoes and banana fruits when fruits are very small and therefore renders physical method inefficient. Other management methods include orchard sanitation, baiting techniques, male annihilation technique (MAT), sterile insect technique (SIT) and post harvest treatment of produce. In Kenya MAT and SIT technologies have not been adopted by farmers due to lack of adequate knowledge and high cost of the technology. Development and application of entomopathogens as part of the integrated pest management offers environmentally benign tool and is an alternative to blanket pesticides spray. Consequently use of entomopathogenic fungi for control of pests is being explored worldwide (Goettel and Johnson, 1992). There are currently over 40 fungal biological control agents (BCA) from division Eumycota, developed from 4 major sub-divisions and registered for use against various pests of medical and agricultural importance (Butt *et al.*, 2001; Lacey and Goettel, 1995). Recently some isolates of *Beauveria bassiana* Bals. and *Metarhizium anisopliae* (Sorok) Metsch (Deuteteromycotina: Hyphomycetes) have shown potential as

biological control agents for management of *Ceratitis* group of fruit flies (Ekesi *et al.*, 2005; Dimbi *et al*., 2003).

1.1 Statement of the problem

The discovery of *B. invadens*, a member of the *B. dorsalis* species complex, considered among the most destructive pests in agriculture which rank highly in quarantine list, symbolizes a major threat to horticultural production in Africa. The problem is compounded by limited use of chemical pesticides, which cause adverse effects to human and beneficial organisms and introduction of the uniform enforcement of strict quarantine and maximum residue levels (MRLs) in the European Union (EU), which jeopardize the lucrative export of fresh fruits from Africa**.** Control of fruit flies in Africa is hampered by the fragmented nature of orchards that contain wild and abandoned cultivated fruits that act as wild reservoirs for a range of fruit flies. This has consequently made the existing fruit fly suppression programs such as sterile insect technique and male annihilation technique very expensive for African governments. Current use of entomopathogens in pest control offers a cost-effective and efficacious alternative to chemical insecticides. In addition entomopathogens have an advantage of safety to non-target organisms and eliminates the use of chemical pesticides or reduces levels of pesticide residue in fruits when used in IPM. This study will select cheap efficacious entomopathogens for the control of *B. invadens* in an effort to salvage problems faced by the horticultural industry.

1.2 Rationale of the study

Control of fruit flies currently rely on use of pesticides in baits or early harvest . *Malathion,* the most commonly used pesticide is a persistent organic toxicant that causes negative ecological impact and cumulative residue effect in the fruit on repeated applications, making it unfit for human consumption. Early harvest method of control is ineffective because *Bactrocera invadens* lays eggs in immature mangoes. The current maximum residue limits and string phytosanitary regulations set export markets jeopardize lucrative export of fresh fruits from Africa. This study, demonstrated feasibility of entomopathogenic fungi to offer green premium that will contribute to IPM to allow for quality production of mango fruit that meet the standards for both domestic and urban export markets.

1.3 Justification of the study

The increasing rate of malnutrition and vitamin deficiency in large sectors of rural and urban populations has lead to an increasing demand on supply of cultivated fruits and vegetables. However, profitable fruit production and export in Kenya is greatly hampered by fruit fly infestation that cause damage of up to 80% at the level of the smallholder contributing to high cost of the fruit. Generally, fruit producers rely heavily on use of chemical insecticides, and over-use of the insecticides leads to accumulation of chemical residue levels on fruits meant for export, which when detected leads to their rejection and loss of trade and revenue. Biological control of fruit flies within the context of IPM is considered feasible. For example previous studies have demonstrated pathogenicities of various isolates of *B. bassiana* and *M. anisopliae* to pupariating larvae and adults of *C.*

capitata, C. cosyra, C. fasciventris, C. rosa and C. anonae in the laboratory (Dimbi *et al.*, 2003; Ekesi *et al.*, 2002). Besides, the studies have demonstrated possibilities of substantially reducing population of *C. cosyra* in the field through use of autoinoculative device impregnated with *M. anisopliae* that consist of cheaply constructed traps and food attractant. No related studies have been done on pathogenicity of *M. anisoplaie* and *B. bassiana* to the recently discovered *B. invadens* (Drew *et al.*, 2005). It is also not known if the effect of fungal infection can reduce fecundity and fertility; and if selected fungal isolate is compatible with food baits for possible exploration in IPM.

1.3 Research Hypotheses

1. Isolates of *M. anisopliae* and *B. bassiana* cannot induce pathogenicity above 80% to adult *B. invadens.*
2. There is no effect of selected entomopathogenic *M. anisopliae* or *B. bassiana* isolates on fecundity or fertility to adult *B. invadens.*
3. (i) There is no difference in persistence and pathogencity of selected *M. anisopliae* or *Beauveria bassiana* isolate with food baits for suppression of adult stages of *B. invadens.*

   ii) There is no difference in compatibility of *B. invadens* with different food baits when combined with *M. anisopliae* or *B. bassiana*.

1.4 Main objective

To identify entomo-pathogenic fungi for management of *B. invadens* on mango.

1.4.1 Specific objectives

1. To determine pathogenicity of selected isolates of *B. bassiana* and *M. anisopliae* to adult *B. invadens.*
2. To assess the effect of fungal infection of candidate isolates on fecundity and fertility of adult *B. invadens.*
3. To assess compatibility, pathogenicity and persistence of the selected isolate with food baits for the suppression of adult *B. invadens* in the laboratory

1.5 Significance and anticipated output

Reduction in mango damage due to *B. invadens* by employing environmentally friendly and affordable management methods using entomopathogenic fungi with food baits.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Generalized life cycle of fruit flies

Life cycle consists of egg, three larval instars, puparia and adult. Color and smell plays a very important role in the choice of site of oviposition. When the right fruit is found, females settle on the surface and pierce the skin of the fruit with the sharp ovipositor and lay eggs underneath the skin. At optimum temperature of 28 ± 1 °C, relative humidity (RH) of 50 ± 8% and a photo period of Light 12 hours: darkness 12 hours (L12:D12), eggs hatch into larvae after 2-3 days producing tiny white larvae which develop from 1st to 3rd instar in the fruit pulp for about 7 days. The larva molts twice during development and during third instar, the maggot jumps out of the fruit into the soil, burrows to the depth of 5-10 cm, and forms a puparium. Pupal duration can vary from 8-14 days depending on the species and temperature. When pupation is complete, the fly emerges and crawls to the surface of the soil. Newly emerged flies are sexually immature and feed for 3-5 days then begin to copulate to commence egg-laying again. Female lays between 300-1000 eggs in lifetime depending on the species and under go 3-15 generations per year depending on climatic conditions and host abundance (Bateman, 1972; White and Elson-Harris, 1992).

2.2 Taxonomic position of fruit flies

Fruit flies belong to the order Diptera and have five sub-families Tephritidae, Trypetinae, Ceratinae, Toxotrypaninae and Dacine, which contains more than 500 genera and 4000

species (Fletcher 1987). The family Tephritidae is further divided into two genera, *Dacus* and *Bactrocera* (Bateman 1972).

2.3 Geo-ecological zones of mango production in Kenya

The area under mango cultivation in Kenya has expanded geographically since 1970 from 500 ha to 15000 ha in 2000 (Ministry of agriculture, Nairobi). The coast province leads in commercial mango production followed by Eastern, Central, Nyanza, Western and Rift valley provinces. Mango grows on a wide range of soils ranging from sandy, loam, black cotton and even murram soil and requires an annual rainfall of 500 -1000 mm, an altitude between 0 and 1200 m above sea level and soil pH value between 5.5 and 7.5. A dry spell is important during flowering stage with temperatures ranging between 20 and 26 ºC for ripening (HCDA, 2002). In the coast, two supply seasons run from November to February and from June to August while in high altitude areas like central province, supply peak is in February and March (HCDA, 2002).

2.4 Varieties of mango grown in Kenya

In Kenya, over 60 different mango varieties have been introduced since 1983 as commercial grade but only about 20 have been successfully established while most of them are still under test. Traditional varieties include Kiitovu, Kimiji, Klaarabu,

Sikio-punda, Mayai, Apple, Ngowe, Boribo, Batawi and Dodo. Selection of popular commercial cultivars is based on resistance to major fungal diseases (anthracnose and powdery mildew). Early cultivars mature from November to February and comprise of Apple, Carabao, Ngowe, Haden, Dodo, Kensington and Zill whereas mid-season

cultivars mature from June to August and consist of Alphonso, Batawi, Heart, Boribo, Golek, Sabre, Madoe, Peach, Sabine, Tommy Atkins, Chino, Mathias, Irwin, Van Dyke, Smith. Carabao variety of mangoes used in field experiment of this study is grown in large scale for both local consumption and export market and has average fruit dimension of 13 cm long by 7.5 cm broad and weight ranging between 220 and 311 g. The fruit grows best in well drained soils and is cultivated in Machakos, Kitui, and along local river banks. All mango cultivars are attacked by fruit flies but apple cultivar is most susceptible. The distribution of fruit flies (*Cerratitis cosyra*, *C. capitata* , *C. rosa, C. anonae ,C. fasciventris* and *B. invadens)* has been reported in almost all mango producing regions in Kenya (Ekesi *et al*., 2006; Copeland *et al*., 2006).

2.5 Control of Fruit flies

Fruit flies exhibit distinctive biological, social and economic attributes that create prospects for a range of control methods (cultural, physical, chemical, behavioral, genetic, biological and IPM).

2.5.1 Cultural, Physical and Chemical Control

Cultural control methods involve continual orchard sanitation through collection of fallen and infested fruit and their subsequent destruction by incineration or deep burying in soil to reduce fly residence population. However in areas where labour costs are low, physical control is used. This involves bagging of large fruits before they reach a suitable stage for fruit fly attack (Allwood 1997) or early harvesting of crops at a stage of maturity at which the fruit is not susceptible to fruit fly attack. However its has been demonstrated that

some *Bactrocera* group of fruit flies such as banana fruit fly *Bactrocera musae* (Tyron), papaya fruit fly *B. papayae* (Drew and Hancock) and *B. invadens* can lay eggs on fruits when fruits are very small. In pawpaw infestation rates by *B. papayae* increases with increasing fruit ripeness while *B. invadens* can also oviposit on green mangoes (Allwood, 1997; Rwamushana *et al.,* 2008). This phenomenon renders the physical method inefficient. Chemical control of adult fruit flies largely relies on use of broad-spectrum organophosphorous insecticides such as the standard malathion. However their use is discouraged due to toxicity to humans and beneficial organisms including pollinators. They are currently replaced by more environmental friendly and superior biological insecticides such as Spinosad, *Scaccharopolyspora spinosa*, which are less harmful to non-target organisms. In contrast to Malathion, spinosad has no phytotoxic activity or cross-resistance.

2.5.2 Behavioral control

It involves manipulation attraction of flies to chemical lure such as a para-pheromone and a phago-stimulatory food attractant made from hydrolyzed protein or combination of fruit volatiles. Attraction is enhanced by use of traps with specific visual cues (yellow, green and red) during detection and control programs. Traps that use male lure are usually based on Steiner trap design, and ammonia bait-traps use McPhail trap (Drew, 1982b). Others include Jackson trap which use adhesive. The para-pheromone Methyl Eugenol (ME) combined with malathion is used in male annihilation technique in high density trapping stations to reduce male population and is considered a universally successful method for eradication of fruit flies. The para-pheromone also occurs in most orchids and

is considered one of the most powerful male attractant of *Bactrocera* species especially *B. invadens, B. zonata* and *B. dorsalis,* but not *B. olae.* It has an attraction range of approximately 500-1000m (Steiner *et al.,* 1965). Eradication approach with ME is only locally applicable in carefully selected single pest infested areas where the fruit industry is well developed and located in naturally isolated area. In protein baits, a phago-stimulatory attractant combined with a toxicant (insecticide) consist of protein hydrolysate that produces ammonia to attract immature adult flies since immature adult flies normally require protein to become sexually mature. The combination is usually added to conventional sprays, to reduce proportion of crop or land area covered with chemical spray droplets and to attract adult fruit fly to the point of application thereby killing it upon ingestion of bait (Steiner *et al*., 1965, Vagas *et al*., 2002). Advanced versions of bait spray are baiting stations consisting of solid paste of the bait and are under developments in the US, Kenya and South Africa.

2.5.3 Genetic control

Sterile insect technique aims at eradicating fruit fly population by releasing sterile males in large numbers to a population to increase chances of their mating with wild females. Due to extensive investment required for its establishment, it is used outside Africa with limited application in Natal Province of South Africa, where it is used for suppression and under certain circumstances, eradication of *C. rosa* and *C. capitata* in production areas (Barnes *et al.,* 2004).

2.5.4 Biological control

Biological control involves the use of naturally existing organisms such as parasitoids, predators and micro-organisms (bacteria, viruses, nematodes and fungi) for control of fruit flies. Hymnopterous parasitoids from the family Braconidae such as *Psytalia concolor* and *Fopius arisanus* (Sonan) are known egg parasitoids of fruit flies; while *P. fletcheti, Diachasmimorpha longicaudata* (Ashmead) and *D. tryoni* are parasites of larvae that have been used in classical releases and inundative release outside Africa for control of melon fly *Bactrocera cucurbitae* (Coquilett), Oriental fly *B. dorsalis* (Hendel), Mediterranean fly *C. capitata* and Queensland fly *Bactrocera Tryoni* (Froggatt) (Wharton *et al.,* 2000; Pena, 1998; FAO, 1998). At ICIPE the parasitoid *F. arisanus* and *D. longicaudata* from Hawaii is being reared for control against *B. invadens* (Samira *et al.*, 2008). Fruit fly predators such as crickets, earwigs, beetles and primates also play an important role in biological control (Allwood *et al.,* 1996). Among microbial control agents, entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* have been used in fruit fly suppression programs in Kenya (ICIPE) and have demonstrated significant levels of reductions in population of *Ceratitis* group of fruit flies (Dimbi *et al*., 2003). No similar information is available for *B. invadens.*

2.6 Systematic position of entomopathogenic Fungi

There are approximately 750 species of entomopathogenic fungi found throughout the five major taxa comprising division Eumycota and exist as obligate facultative pathogens (Ainsworth, 1973). Within the five fungal subdivisions the highest numbers of

entomopathogens are found in sub-division Deuteromycotina followed by Zygomycotina and Mastigomycotina.

2.6.1 Subdivision Mastigomycotina

2.6.1.1 Class Oomycetes

The genus *Lagenidium* (Oomycetes: Lagenidiales), particularly *L. giganteum* Couch, *and L. chapmani* are parasitic to a wide range of anopheline and culicine mosquito larva (Kerwin *et al*., 1997).

2.6.1.2 Class Chytridomycetes

The genera *Coelomomyces* (Chytridiomyces: Blastocladiales) are obligate parasites that alternate their hosts between mosquito larvae and aquatic crustaceans e.g. *C. psorophorae* Couch, *C. stegomyiae* and *C. dodgei* Couch and Dodge.

2.6.2 Subdivision Zygomycotina

2.6.2.1 Class Zygomycetes

Species of the order Entomophthorales are among the most promising group of biological control agents against insect pests (Roberts and Humber, 1981; Watson *et al*., 1996). Entomophthorales infect approximately 32 families of insects across several orders and contain the genera *Entomophthora, Massospora, Zoophthora, Neozygites, Pandora, Furia, Erynia* and *Conidiobolus*. Entomophthorelean fungi are obligate pathogens that do not produce toxins of importance during disease progress but often cause high infection levels and epizootics in insects (Carruthers *et al*.,1985; Mullens *et al*., 1987; Mullens, 1989; Watson *et al.,* 1996). For example, pathogenicity of ***E. miamaga*** Humber and *E.*

grylli to gypsy moth *Lymantria dispar* and a diverse group of acridids respectively causes disease epizootic. The genera *Massospora* Peck is however restricted to gregarious cicadas (Homoptera: Cicadadae). *Zoophthora radicans* Batko is a spp complex. Within the complex are *Z. phalloids* and *Z. phytonomi* Batko which infect aphids and Hypera species (Coleoptera: Curculionidae) respectively. *Neozygites* Witlaczil e.g. *N. fresenii* (NovaKowski) and *N. floridana* are pathogens of aphids and tetranychid mites. The genera, *Pandora* Humber, *P. delphacis* (Hori) Humber, *P. neoaphidis* infect diamond back moth, *Plutella xylostela*, plant hoppers and aphids respectively.

2.6.3 Subdivision Ascomycotina

2.6.3.1 Class Pyrenomycetes

Entomopathogenic ascomycetes belong to a very narrow range of genera in the Claviceptales. More than 300 spp of *Cordyceps* within the order Claviceptales have been reported pathogenic to five major insect orders. Besides, *Claviceps* and *Cordyceps* (Fries) species are also known to be pathogenic.

2.6.3.2 Class Ascospheaeromycetes

Ascosphera species are the causative agents of chalk-brood, a serious disease affecting bee larvae (Hymenoptera). The major species are *A. aggregata* Skou, and *A. apis* which infects leaf-cutting bees (Megachilidae) and honey bees (*Apis mellifera*) respectively (Humber, 1997).

2.6.4 Subdivision Deuteromycotina (Fungi imperfecti)

It is reported to contain more than 40 genera that infect insects across various orders. A number of fungi in this class cause muscardine diseases of insects (Inglis *et al*., 2001).

2.6.4.1 Class Hyphomycetes

Several genera that belong to the Hyphomycetes are entomopathogenic to several insect orders. The following genera are reported as examples.

Hirsutella: Approximately 35 species have been described with several species affecting arthropods mites, and leaf hoppers; *H. thomsonii* (Fisher) is used in control of citrus rust mite and cassava green spider mite.

Paecilomyces: Approximately 14 entomogenous spp exist. *Paecilomyces furinosus* (Holm) Brown and Smith, *P. fumosoroseus* (Wize) and *P. tenuipes* (Peck) Samson are used as BCAs. Others include *P. lilicanus* and *P. javanicus.*

Nomurae: The species are pathogenic to several economically important Lepidoptera. *Nomurae rileyi* is pathogenic to nearly all developmental stages of major *lepidotera* pests including soya bean pests. Both *Nomurae rileyi* and *N. anemonoides* cause natural epizootics *cause natural epizootics in Noctuidae* (Mohamed *et al*., 1977), but *N. atypocola (Yasuda)* is pathogenic mainly to spiders.

Verticillum lecani: (Zimmerman) Viegas The genera is a species complex pathogenic to all development stages of Arachnida and insecta especially scale insects, aphids and

whiteflies. *Verticiliiium lecani VERTALI*® is used in control of aphids and white flies on protected turf crops such as cucumber, tomatoes, egg plant, lettuce, beans, and ornamentals. In Kenya *V. lecani* produced by Dudutek is currently under trial against scale insects, aphids.

Beauveria species*:* The most important examples are *Beauveria bassiana* and *B. brongniartii.* Conidiogenous cells are usually densely clustered, whorled or solitary with globose flask-like base and denticulate apical extension bearing one conidium per denticle. Methods for production of *Beauveria* conidia on solid substrate are numerous with variation in techniques. They are commercially produced by several companies outside Africa for control of Coleoptera and Lepidoptera, Homoptera, Orthopterans occurring on high value horticultural crops like coffee, flowers, vegetables, wheat. Formulations such as oil, clay granules, wettable powder are prepared under commercial product labels like Agobiocontrol5®, Boverin Mycocide-gha from *B. brongniartii* (Ferron, 1981; Jaronski and Goettel, 1997). In Africa use of *Beauveria* species and several entomopathogens is limited to experimental trials on high value export and traditional crops as their research on efficacy development progresses towards registration of products. Within the past few years, a biopesticide company based in Kenya, (Dudutech) conducted several field trials using entomopathogens on pests of commercial vegetables.

Metarhizium: The *Metarhizium* genus has global distribution that includes several degrees of pathogenicity towards insects. Three main species are generally recognized,

M. anisopliae (Metschnikoff) Sorokin, *M. flavoviridae* Gams and Rozsypal *M. album* Petch. *M. anisopliae* is separated into three main varieties: var. *anisopliae,* var. *majus* and var. *acridium.* The conidiophores are broadly branched (candelarum-like), densely intertwined; conidiogenous cells arranged in dense hymenium; chain-forming conidia are usually aggregated into prismatic or cylindrical columns or solid mass of parallel chains making it easy to harvest conidia with minimal amount of mycelia. The genus is responsible for the green muscardine disease and is pathogenic to over 200 species. Agobiocontrol-Metarhizium-50® *M. anisopliae* product is used for control of Lepidoptera, Coleoptera and Homoptera on flowers, vegetables and potatoes; while Green Muscle ®, *M. anisopliae* var. *acridum* (strain IMI 330189) is a product produced by Biological Control Products of South Africa, is used for control of grass-hoppers and desert locust *Schistocerca gregaria* (Lomer *et al.*, 1997). Several other isolates of *M. anisopliae* at ICIPE have been developed and are used for control of thrips, termites and tsetse flies (Ekesi *et al*., 2000, Milner 1998).

2.7 Mass Production of Entomopathogenic fungi

Mass production is technically a professional method producing pathogen for classical, augmentative or inundative field-pathogen releases. Entomopathogenic hyphomyceteous fungi are facultative micro-organisms and can grow as submerged cultures and aerobically on solid media, producing blastospores or conidia respectively (Jenkins *et al*., 1998; Ferron, 1981; Roberts and Humber, 1981). Their production in large scale is usually based on a standard-two-stage (diphasic) mass production batch system that combines benefits of submerged/aerially produced conidia (Lomer *et al*., 1997). In the

first stage, fungal biomass is produced under optimized temperature and aeration and pH, following inoculation in liquid medium to support higher yield of blastospores for subsequent inoculation onto solid substrate. Large mass production units use cheaply available raw materials like molasses, waste brewers yeast and corn steep liquor as starter culture, while small production units use undefined commercial media consisting of glucose, peptone and yeast extract to serve as sources of carbohydrate and nitrogen respectively (Jenkins and Prior, 1993; Cherry *et al*., 1999)). Successful mass production unit provide reliable and efficacious quality control tests based on product bioassay that measure biological activity for each batch and determine $LC_{50.}$

2.8 Modes of Infection of Entomopathogenic hyphomycetes in insects

The most common route of infection for entomopathogenic fungi is via the external integument although infection via the digestive tract and spiracles is possible. Infective conidia develop through host cuticle depending on availability of favorable conditions of relative humidity and temperature within insect cuticular microclimate. Disease development of entomopathogenic fungi is divided into several steps:

2.9 Attachment of fungal conidia to the insect cuticle

2.9.1 Hydrophobic interactions

Insect cuticle acts as primary barrier fungal propagules. Both conidia and insect cuticle are hydrophobic causing an initial passive interaction between them. Several factors are presumed to contribute to hydrophobic interactions; especially structure of cuticular layer and hydrophobic proteins within the rod-let layer of the cell wall. Hydrophobic proteins

produce hydrophobins and facilitate initiation of non-specific adsorption of conidia onto surface cuticle and subsequent formation of fungal appressorium and extrusion of a sticky mucilage layer (St. Leger *et al*., 1992a).

2.9.2 Conidial Germination on epicuticular layer

Most entomopathogenic fungi require exogenous nutrients, ions, free water (available from the outermost epicuticular layer of the host cuticular folds, interguments and microflora for germination and are adopted to utilize alkanes and lipids as source of carbon. The cuticle also contains other compounds, primarily amino acids and amino sugars which function as nutrient sources for fungal germination. The layer contains complex sugars (e.g. acetylglucosamines), secondary alcohols, glucose, chitin, starch, fatty acids and nitrogen reserves that act as substrates and are enzymatically degraded by enzymes (lipases, esterases exoproteases, chitinases) produced from fungal cell walls (Butt *et al.,* 1998; Hajek and St. Leger, 1994; St. Leger, 1993; Ferron, 1985).

2.9.3 Procuticular fungal Penetration

Germination and penetration by fungus are primarily affected by physical parameters attributed to the cuticle such as its hardness, degree of sclerotization, chitin content and availability of water and nutrients. Principally, penetration of the cuticle during germination requires appropriate RH (>90%), temperature and ions. In order to penetrate the cuticle during germination, fungi use appressoria to attach firmly to the cuticle together and use physical pressure plus a battery of enzymes (proteases, chitinases, glucosaminases and lipases) which initially hydrolyze cuticular proteins followed by

chitin and allow projection of a narrow infection peg into the cuticle (St Leger *et al*., 1986a; 1989). *Metarhizium anisopliae* additionally, produce hydrogen peroxides which bleach melanin of insect cuticle and cause softening thereby facilitating hyphal penetration. This is followed by production of extra cellular chitinases, Nacetyglucosaminases produced by the fungi. Chitin fibrils are surrounded by protein sheath and therefore, initially enzymatic degradation starts with protein and subsequently chitin (St Leger *et al*., 1986a).

2.9.4 Immune Responses

2.9.4.1 Humoral immune response

Cuticular phenoloxidase, lectins, peptides and proteins act in humoral response and in defense reaction implicated in melanin patches on wounded cuticle and infection sites hampering fungal infection. However, cuticular physiological process has not been well understood.

2.9.4.2 Cellular immune responses

The ß-1-3 glucans produced by fungal cell walls activates cellular immune response causing encapsulation and phagocytosis. Haemocytes and plasmocytes aggregate at the epidermal area of fungal penetration through cuticle suggesting chemical signal release into haemolymph during initial penetration process; while granulocytes adhere to entrapped conidia to cause lysis. The entrapped conidia become melanized; and if the growth of fungi in the haemolymph out-competes cellular immune response in the haemolymph, free living blastospores multiply. While in the haemolymph, blastospores

produce toxins insecticidal cycloeptides, (dextrusins; beauveracin, bassianolide and oosporein), which work by opening Calcium channels in muscle membranes and induce tetanic paralysis (Samuels *et al.*, 1988).

2.10 Post-penetration events

Once the cuticle has been breached by the penetrant hyphae, it develops as blastospores within the haemocoel and use water, carbohydrates, proteins and amino acids, which serve as a source of carbon and nitrogen for its growth. The parasitic phase ends with depletion of nutrients, invasion of organs, production of toxins and death of host. Under increased RH conidiophores grow out from hyphal bodies and emerge through less resistant portions of the exoskeleton, producing terminally borne conidia to the exterior of host cuticle.

2.11 Factors influencing fungal efficacy on insects

Fungal efficacy is mainly influenced by abiotic and biotic factors. Abiotic are to availability of water, suitable temperature, length of dew periods, microclimate, nature of canopy, rainfall pattern while biotic factors involve pathogen strain, physiological state of host, nutrition, defense mechanism, cuticular and epicuticular micro-organisms.

2.11.1 Abiotic Factors

Under suitable conditions of relative humidity, temperature can influence the rate of infection. Tropical isolates of *M. anisopliae* and *B. bassiana* have demonstrated high groth and infection at optimum temperatures between 20-25 °C; inhibition of growth at

temperatures above 30 °C and cessation at 37 °C. Fargues (1997b) demonstrated ability of four isolates of *M. anisopliae* var. *acridum* to induce 98-100 % mortality in *S. gregaria* between 25 °C and 30 °C, but when the same pathogen was applied to *S. gregaria* kept at 40°C mortality was 0%. Acridids are able to thermo-regulate and elevate their body temperatures higher than ambient by intercepting solar radiation and consequently raising their body temperature upto approximately 40°C which decreases mycosis and influence disease development in *B. bassiana* and *M. anisopliae* by 45% and 80% respectively (Inglis *et al*., 1996a).

Relative humidity is a crucial requirement for fungal sporulation, spore germination, conidiogenesis of infected cadavers and initiation of fungal epizootics (Fargues *et al*., 1997).

The Ultra violet-B (UVB) portion of solar radiation spectrum (295-320nm) is the most detrimental to germination of conidia. It causes rapid inactivation of propagules, hyphal bodies, and hypha to all taxa of hyphomycete fungi, resulting to their short period of persistence when exposed to sunlight (Fargues *et al*., 1996). Inglis *et al*., 1993; 1997a) determined the influence of sunlight on susceptibility of caged grasshoppers to *B. bassiana* under varied UVB radiation of protected environment and those exposed to full sunlight radiation while maintaining constant temperature and RH. Results showed conidial survival was significantly enhanced with slightly higher prevalence and more rapid development of disease in UVB-protected environment (43%) than in cages exposed to full sunlight. Variation to UVB tolerance between isolates and specific groups of fungi has been reported, e.g. in laboratory exposure experiment using artificial UVB radiation against conidia of *M.anisopliae var. flavoviridae, B. bassiana, M. anisopliae*

and *P. fumosoroseus* incorporated with solar blockers showed that conidia of *M. anisopliae* var. *flavoviridae* were more resistant to artificial light (295-1100 nm) followed by *B. bassiana*, *M. anisopliae* and *P. fumosoroseus* (Fargues e*t al*., 1996). It has been proved that certain micro-organisms (*Micrococus leutus*) use the solar spectrum irradiation of higher wavelengths (<320-384 nm) to stimulate photo reactivation, a phenomenon by photolyase enzyme that splits thiamine dimmers, bonded by UVB into monomers and revert nucleotides to their original state, no such information is available regarding fungal entomopathogens.

2.11.2 Biotic Factor

Factors related to insect host like age, nutrition, population densities induce stress and predispose insects to infection. High densities also increase chances of horizontal transmission and disease epizootics with early insect developmental stages being more susceptible. During infection process**,** two types of diseases are recognized, non-infectious caused by poisons, physical injuries, abiotic factors; and infectious diseases caused by pathogens. The term pathogenicity denotes the potential of a micro-organism to produce disease when applied to groups or species of hosts (Stainhaus, 1975). Agostino Bassi, (2000) was the first scientist to demonstrate the pathogen theory of disease by proving that *B. bassiana* was the causative agent of the white muscardine disease on silk moth using mycosis as confirmation. Individual isolates can exhibit pathogenicity to restricted host range e.g. *Aschersonia aleyrodis* infect white flies and soft scales; and *Nomuraea rileyi* almost exclusively infects lepidopterans. In contrast, species such as *Metarhizium anisopliae, Beauveria bassiana and Z. radicans* have much

wider host ranges across numerous orders of insects (Moore and Prior, 1993), implying presence of a diverse assembly of genotypes that comprise of species complexes.

2.12 Fungal Persistence in the Environment

Stability of propagules during storage, ability to apply pathogen successfully to the target insect and knowledge of pest-pathogen interaction in the field is useful in determining persistence of propagules in the field. In order to increase propagules persistence in the field, humectants and UV protectants are incorporated during development to protect propagules from solar radiation and low RH (Inglis *et al.*, 1996d). For example optical brighteners like stilbene, clay, tinopal; dry formulations and oil-based formulations e.g.ondina, shellsol, peanut oils; are added to increase persistence of propagules in the environment (Alves *et al*., 1998; , Inglis *et al*., 1995*a;*1996d).

2.13 Methods of Microbial control of insect pests using fungi

2.13.1 Classical Control

Classical control is aimed at permanent establishment of an exotic pathogen in a new area to control pest population. The exotic pathogen introduced should have the potential to persist in the environment and spread from sites of release. For introduction of exotic pathogens to occur, a candidate pathogen is usually obtained from the area where introduced pest is indigenous. Classical biological control program are suitable for stable ecological systems such as forests where long-lived stages of entomopathogen can persist as compared with annual crops in agriculture and systems that contain low host densities to retain the pathogen in population. Successful programs conducted with the concept of

CBC of entomopathogenic fungi include application of the fungus *Entomophaga maimaiga* against forest defoliator *Lymantria dispar* (Hajek *et al.*, 1995). These applications demonstrate epizootics on host population by production of many infectious propagules that are re-distributed into the host population causing high mortalities.

2.13.2 Inoculative release

For inoculate release, it is assumed that if some minimal amount of inoculum is introduced, the organism will then be transmitted in the host population on its own to have an impact on host population density. This method is suitable for fastidious organisms or those that are difficult to produce in vitro and require little inoculum for release (usually produced in field related or laboratory reared hosts). The method is appropriate for introducing pathogens that attack hosts leaving predominantly in cryptic habitats (e.g. *rhinoceros* beetle) or locations hard to reach using standard spray applications. Fungal inoculums can be released in a many forms e.g. living-infected hosts, spore filled cadaver or release of resistant stages of fungi applied as dust or sprays directly onto the substrates that hosts eat or in which they live as in the case of soil dwelling pests (Papierok and Hajek, 1997; Bidochka *et al*., 1996). It is a more useful method of release if conidia are fragile and short lived. It has been used to establish entomophthoralean species and *Zoophthora radicans* in aphid infested areas outside Africa (Milner, 1982, 1985). Cultures of these fungi can be grown on artificial media and then inverted over target pest-infested plants in the field so that forcibly dislodged conidia can land on susceptible host. The infected insects kill hosts on plants while air-borne conidia on mobile-infected insects cause subsequent spread of pathogen.

2.13.3 Inundative release

Inundative introduction consists of releasing large quantities of inoculum in larger areas for immediate control without waiting for the pathogen to increase by its own in the environment. This practice is only possible with pathogens that can be mass produced and for hosts that live in habitats where they are contacted by broadcast application as in the case of *L. giganteum* against larval and pupal stages *Aedes inpromaculis* and *Culex farsalis* (Lacey and Undeen, 1986).

2.13.4 Auto-inoculating Devices

It is among the potential methods used for release and distribution of entomopathogens. Auto-dissemination method uses adult hosts for transmission purposes through use of lures such as pheromones or baits that emit specific volatile cues and attract hosts into source of entomopathogen, from which they are contaminated and act as vectors by disseminating pathogen to other members of the target pest population. The technique employs small quantities of BCA thereby minimizing production cost and storage space and plays a vital role in resistance management. It also has an advantage of reduced harm to natural enemies since the pathogen is target-specific. The inoculum is usually introduced in designed trap that protects propagules from damaging effects of UV radiation and increase its persistence in the environment. Auto-inoculators may be used to initiate earlier epizootics during low pest population as opposed to normal epizootics that need large host densities which occur when it is too late in the season to restrict damage below economic threshold (Vega *et al.,* 2000; Lacey and Kaya, 2000). Successes in use of autoinoculators include those with sex pheromone- lures, phago-stimulatory

attractants and entomopathogens used as in control of diamondback moth *Plutella xylostella,* Japanese beetle *Popilla japonica,* tse-tse fly *Glossina* spp. and *Ceratitis* spp. of fruit flies using *Zoophthora radicans, B. bassiana, M. anisopliae* respectively (Dimbi *et al*., 2003; Maniania *et al*., 2002; Klein and Lacey, 1999; Furlong *et al*., 1995; Pell *et al*., 1993ab. Recent studies by Dimbi *et al* (2003) on evaluation of infection rate of IC20-impregnated dry cheese cloth previously soaked in molasses and held above Nulure in a trap and exposed within mango canopy to target adult *C. capitata,* show promising results with high levels of fly attraction, high fly mortalities and drastic reduction in fecundity and fertility during a period of 4 weeks when conidial viability was above 70%.

2.14 Safety of Microbial insecticides (MI)

The basic rationale in the development and use of MIs is associated with their restricted host range and reduced harm to non-targets (Goettel *et al*., 2000, Vestergard *et al.,* 2000). Inoculative release of fungal pathogens is considered safe and effective when applied to target pests initiating epizootics in host population and remaining in the host environment to subsequently infect other generations of the insect (Hajek e*t al.,* 2000). Infection studies for mammalian safety screen on mice with commercial strains of *M. anisopliae* and *Beauveria* showed no systemic infection via inhalation and subcutaneous; long-term feeding studies on rats and birds; intraocular injection in rabbits and oral dosing (Shadduck *et al*, 1987; Goettel and Jaronski, 1997). However both isolates showed teratogenic effect and decreased cardiac output on silverfish. *Metarhizium anisopliae* var. *acridum* showed extreme toxicity and hyperallerginecity to mice and pulmonary mycetoma to fringe toed lizard *Acanthodactylus dumerilion* upon prolonged treatment

(Goettel and Jaronski, 1997). Reports on effect of *B. bassiana* and other potential fungal control agents to beneficial non-target invertebrates show generalized pathogenicity to honey bee, *Apis mellifera;* commercial silk moth *Bombyx mori* and Hymnopterous parasitoids *Bracon hebetor* and *Apoanagyrus iopez* (Damfa and Van Der Valk, 1999; Goettel, 1996, 1990). But no records exist on natural epizootics caused by the fungi allowing low risk to the non-target insects in a field situation. MIs can be applied within short pre-harvest interval (PHI) to salvage crop damage at late stages in many agricultural systems without risk of residue unlike chemical insecticides, which require long PHI to allow dissipation of residue prior to consumption. At both situations exposure risk is experienced during application and mass production. This has prompted current environmental safety to be forged through development of IPM technologies that selectively use integrated fungal control agents with other control strategies like chemical control, physical, and pest behavior.

CHAPTER 3

3.0 MATERIALS AND METHODS

This chapter contain details of all materials and methods used in bioassays that: (1) evaluated pathogenicity of 24 isolates of *M. anisopliae* and *B. bassiana* to adult *B. invadens*, (2) assessed effect of selected isolate (*M. anisopliae* ICPE 20) on fecundity and fertility of female *B. invadens* and (3) evaluated, i) viability of *M. anisopliae* ICPE 20 spread on 3 different food bait formulation and exposed under mango canopy in the field for a period of 28 days, ii) resident time adult *B. invadens* spent on contaminated bait on bait and iii) pathogenicity of the *M. anisopliae*-treated baits to adult *B. invadens* in the laboratory on day 0, 7, 14, 21, 28.

3.1 Insect source and rearing conditions

Adult *B. invadens* (Plate 3.1) were obtained from mass rearing stock maintained at ICIPE. Larvae were reared on a carrot/sugar based artificial diet and adult flies maintained on a sugar and yeast hydrolysate based artificial diet (Ekesi *et al*., 2005) (USB Corporation, Cleveland, Ohio, USA). The insects had been maintained on the artificial diet at 26 ± 2 ^{o}C, relative humidity (RH) of 45%, photoperiod of 12 hours light and 12 hours darkness (L12: D12).

3.2 Source of fungi and maintenance of fungal cultures

The 24 fungal isolates of *M. anisopliae* and *B. bassiana* (Plates 3.2 and 3.3) used in this study were obtained from the ICIPE Microbial Germplasm (Nairobi) and from pathogen survey carried out during the study (Table 3.1). All the fungi were maintained on

Sabouraud Dextrose Agar (SDA) in Petri dishes and incubated at ambient temperatures of 26 ± 2 ^{o}C for 2 to 3 weeks in the laboratory before being used for pathogenicity test. Solid substrates are preferred for production of high yield of aerial conidia of entomopathogenic fungi, although strands of broken mycelia found in the mixture can also be infective.

3.2.1 Methods for testing conidial viability

Viability of the conidia were tested by scrapping the surface of the 2-3 weeks old fungal culture and suspending the inocula in 15 ml of sterile 0.01% Triton X-100 (v/v) in universal bottle containing glass beads that measured 3 mm diameter to obtain a stock solution. The suspension was then vortexed for 5 minutes to obtain homogeneous suspension of conidia. A final concentration of 3 x 10^{6} spores per ml was prepared by diluting from the stock and quantifying with Bright Line® Improved Neubauer Haemocytometer (Buffalo, New York, U.S.A.). A volume of 0.1 ml of conidial suspension was aliquoted and spread plated onto clean SDA plates. A total of 5 plates were cultured for each isolate and tested for viability. Two replicates were made, each from respective plate of the same isolate. The plates were incubated at 26^{o} C ± 2 (Lab-line Imperial incubator, Melrose, ILL) for a period of 16-18 h followed by fixing with lacto-phenol cotton blue to terminate fungal growth. Six sterile slide cover slips were placed on each plate and six viability observations recorded from each plate. Viability was determined by counting a total of 100 conidia (germinated and non-germinated). A total of five plates were examined for each isolate and mean percentage germination was

determined. Propagules were considered viable if the germ-tube length were two times the diameter of conidia.

3.2.2 Effect of time intervals on pathogenicity of *B. invadens* by *M. anisopliae*

Two fungal isolates of *M. anisopliae* (ICIPE 20 and ICIPE 60) were used for preliminary studies of a bioassay. Conidia was scrapped from 2-3 weeks old culture using wire loop while working under laminar flow hood (Sterigard, USA). Measurement of 0.3 g of conidia was obtained using a balance (Mettler AE 166, Switzerland) and conidia evenly spread onto a clean inoculating chamber using a spatula. The chamber consisted of a cylindrical plastic tube (9 x 13 cm) lined on the inside with velvet material. One end is fitted with a nylon mesh and the other a perforated hole (5 mm diameter) that served as a source of light for insects (Plate 3.4) (Dimbi *et al*., 2003). Two hundred adult flies aged between 7-10 d were picked from the culture. Five flies were introduced into the inoculating chamber for a period of 30 seconds, 1, 2, and 3 minutes. Infected flies were removed from the chamber and vortexed thoroughly for 5 minutes to wash off conidia from the body followed by determination of spore concentration per fly using Bright Line® Improved Neubauer Hemacytometer as described in section 3.2.1. Each treatment was replicated 5 times. Control insects were exposed on clean chamber (without fungus) and similar exposure times to the corresponding treatment. Mortality was observed and recorded after every 24 hours for a period of 5 days and confirmation of mycosis recorded between day 2 and 5 of incubation.

3.2.3 Effect of *M. anisopliae* and *B. bassiana* isolates on mortality of *B. invadens*

3.2.3.1 Experimental design

The experiment was structured to have 20 flies per replicate with 5 replicates per isolate. Twenty flies were picked at random and infected with the 24 isolates of *M. anisopliae* and *B. bassiana*. Cages were arranged in a completely randomized design in the experimental room. Mortality was measured as a percentage of dead flies within a replicate. Single factor analysis of variance was used to compare means, where mortality was the variable of interest and virulence the factor to be assessed.

3.2.3.2 Experimental procedure

Adult *B. invadens* were contaminated using velvet material impregnated with 0.3 g of dry conidia of *M. anisopliae* or *B. bassiana* that covered inoculating chamber prepared as explained in section 3.2.2. Conidia from each plate were spread on individual contaminating chamber to avoid pseudo-replication. From a cohort of 1000 flies aged between 7 and 10 days, 20 adult flies, were picked and introduced into the contaminating chamber for 30 seconds. Thereafter, contaminated insects were transferred into Plexiglas cages (15 x 15 x 20 cm) and fed on a mixture of sugar and commercial enzymatic yeast hydrolysate at a ratio of 3:1. Water was supplied in falcon-tube lids filled with pumice granules. Each isolate was replicated 5 times with 20 insects per replicate. Control insects were exposed to clean chamber (without fungus) for the same time period as in the fungus treated flies. The flies were then maintained at ambient temperatures. Fly mortality was observed daily and recorded for a period of five days. In order to score for mycosis, dead insects were surface sterilized by washing in 1-2% sodium hypochlorite

for 2 min; followed by 70% alcohol for 1 min and rinsed thrice in sterile distilled water. Cadavers were then incubated in 9 cm Petri dishes lined with moistened filter paper. Mycosis on cadaver was observed and recorded after 3-5 days. Confirmation of mycosis was demonstrated by presence of fungal growth and sporulation on the surface of the cadaver. A total of 24 isolates of *M. anisopliae* and *B. bassiana* were tested for pathogenicity against *B. invadens*

3.2.4 Assessment of mean number of conidia picked per fly upon exposure to four different isolates of *M. anisopliae*

In this experiment, 0.3 g of dry conidia were scrapped from 2-3 weeks old cultures of *M. anisopliae* and evenly spread onto clean inoculating chamber (Dimbi *et al*., 2003). From a cohort of 1000 flies of mixed sexes, five flies, aged between 7 and 10 days were picked from the colony and introduced into the inoculating chamber for 30 seconds. Using a fine forceps, each fly was picked and transferred into 1 ml Eppendorff tube that had 2 glass beads and 0.5 ml of sterile solution of 0.01% Triton X-100 in water. The flies were vortexed thoroughly for 5 minutes to wash off conidia from the body followed by determination of spore concentration per fly using the methodology in section 3.2.1. Four isolates of *M. anisopliae* were tested with 5 replications per isolate.

3.3 Maintenance of virulence of *Metarhizium anisopliae* isolate ICIPE 20

Based on the pathogenicity and virulence bioassay, *Metarhizium anisopliae* isolate ICIPE 20 was selected to study its sub-lethal effects on reproduction of *B. invadens*. Its virulence was maintained through passage on adult *B. invadens*. The fungal isolate was grown and maintained on Sabouraud Dextrose Agar (SDA) in 9 cm Petri dishes (section

3.2). Similar procedures of viability test and infection are explained in section 3.2.1 and 3.2.3.

Table 3.1: Fungal isolates tested for pathogenicity against adult *Bactrocera invadens* and listed by ICIPE's accession number; original host of isolation; country of origin followed by locality and year of isolation.

Fungal Isolate	Accession number	Substrate/ Host	Country	Locale	Date of isolation
M. anisopliae	ICIPE 18	soil	Kenya	Mbita	1989
	ICIPE 55	soil	Kenya	Embu	2005
	ICIPE 95	sand fly	Kenya	Kitui	2005
	ICIPE 387	*Forfificula senegaliensis*	Kenya	Mai-maihu	2007
	ICIPE 402	Homoptera	Kenya	Shimba Hills	2007
	ICIPE 41	soil	Lemba	Zaire	1990
	ICIPE 7	*Amblyomma variegatum*	Kenya	Rusinga island	1996
	ICIPE 60	soil	DRC	Matete	1990
	ICIPE 69	soil	DRC	Matete	1990
	ICIPE 43	soil	Kenya	Meru	2005
	ICIPE 62	soil	DRC	Kinshasa	1990
	ICIPE 49	soil	Kenya	Mt Kenya	2005
	ICIPE 23	*Ornithacris cavroisi*	Niger	Niger	1998
	ICIPE 69	soil	Zaire	Matete	1990
	ICIPE 58	soil	Kenya	Kericho	2005
	ICIPE 53	soil	Kenya	Kitui	2005
	ICIPE 295	soil	Mauritius	unknown	2005
	ICIPE 20	soil	Kenya	Migori	2002
B .bassiana					
	ICIPE 33	*Rhepicephalus appendiculatus*	Kenya	Rusinga Island	1996
	ICIPE 34	soil	Kenya	Mbita	2003
	ICIPE 273	Soil	Kenya	Mbita	2006
	ICIPE 603	Hymenoptera	Kenya	Taita	2007
	ICIPE 284	soil	Mauritius	unknown	2005
	ICIPE 280	soil	Mauritius	unkown	2005
	ICIPE 282	soil	Mauritius	unkown	2005
	ICIPE 303	soil	Mauritius	unknown	2005

Plate 3.1: Adult *Bactrocera invadens*

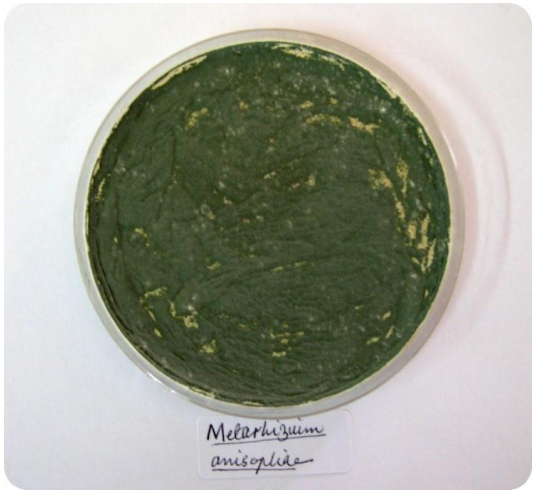


Plate 3.2: *Metarhizium anisopliae* isolate growing on Sabouraud Dextrose Agar

Plate 3.3: *Beauveria bassiana* growing on Sabouraud Dextrose Agar

Plate 3.4: Inoculating chamber

Plate 3.5: Inoculating chambers spread with spores of *B. bassiana* and *M. anisopliae*

3.4 Assessment of fecundity, mortality and hatchability

3.4.1 Experimental design for fecundity and pathogenicity

Treatment material was 0.3 g conidia of *M. anisopliae* isolate ICIPE 20 harvested from 3 culture plates spread evenly onto each inoculating chamber. Experimental unit consisted of 10 pairs (male and female) of *B. invadens* aged 10-14 days old, sampled randomly and treated as a replicate. This was replicated 3 times. Each pair of flies in a replicate served as sampling unit, and was separated into individual cages with a total of 10 sampling units per replicate. Controls consisted of equal number of insects exposed to clean chamber without fungus, replicated 3 times and sub-sampled as above into individual cages. Variables were egg fecundity and mortality. Data was collected on day 1, 2, 3, 4 and 5. T-test was used to compare means between controls and treatment .

3.4.2 Procedure for fecundity

Dry spores of *M. anisopliae* (ICIPE 20) (0.3 g) were harvested from the surface of 3 weeks old sporulating culture and evenly spread onto the surface of a velvet lining the inner wall of a cylindrical plastic tube that had the bottom removed and fitted with a nylon netting as explained in section 3.2.3. From a population of 600 adult *B. invadens* aged between 10-14 days, 10 pairs (male and female) adult *B. invadens* were transferred into the cylindrical tube and allowed to walk on the surface of the contaminated velvet for a period of 30 seconds. Control insects consisted of 10 pairs (male and female) exposed to clean velvex chamber (without fungus) for 30 seconds, replicated thrice. After exposure, the flies were removed from the tube and a pair (male and female) transferred to a 15 x 15 x 15 cm transparent Plexiglas cage containing a ripe apple mango dome

(approximately10 cm long and 9 cm diameter), which was used as oviposition substrate (Plate 3.8.). To make the dome, a mango was cut into two halves and the pulp and seed removed and the skin was then spiked with several small holes using an entomological pin (38 mm long, 0.3 mm diameter) to facilitate oviposition. The dome was then placed over a 9-cm Petri dish lined with moistened filter paper and later transferred to the plexi glass cage.. Control insects consisted of equal number of insects exposed to clean chamber without fungus. The room was maintained at a temperature of 26 $^{\circ}$C ± 2 and RH of 65% ± 2. The insects were fed on diet consisting of 3:1 mixture of sugar and enzymatic yeast hydrolysate. Lids of falcon tubes filled with water and pumice granules served as water source throughout the experimental period.

3.4.3 Assessment of Fecundity

In order to evaluate the effect of fungal infection on fecundity, eggs laid per individual fly were collected from the mango dome after every 24 h intervals from day 1 to 5 post-infection. Using a fine camel's hair brush, the eggs were gently transferred onto a 5 x 5 cm water soaked black cotton cloth placed in a Petri-dish (9 cm diameter) (Plate 3.9). Using a dissecting microscope, total number of eggs laid per fly per day were counted after every 24 hours for a period of 5 days. The mango dome was changed after every 2 to 3 days depending on the rate of drying and to minimize mould formation. Each treatment consisted of 10 females per treatment replicated 3 times.

3.4.4 Pathogenicity of *M. anisopliae* on gravid females

Effect of *M. anisopliae* (ICIPE 20) on pathogenicity of gravid adult *B. invadens* aged between 10-14 days infected with spores was assessed from subsequent flies that were used on fecundity studies as explained in the earlier procedure of this chapter (section 3.2.3). Fly mortality was monitored as explained previously in section 3.2.3. Control insects (10 flies) were exposed to clean velvex chamber (without fungus) for 30 seconds. After exposure, the flies were removed from the tube and transferred individually to a 15 x 15 x 15 cm transparent Plexiglas cage containing a ripe apple mango dome (approximately10 cm long and 9 cm diameter). Effect of *M. anisiopliae* ICIPE 20 was assessed by taking mortality records after every 24 hours as mentioned in the previous procedures in section 3.2.3.

3.4.5 Assessment of hatchability

3.4.5.1 Experimental Design

Experimental unit consisted of a replicate of 10 egg masses (Each egg mass was laid individually by *M. anisopliae* treated flies in section 3.4.2) and incubated at room temperature (26 ± 2 $^{\circ}$C and a photoperiod of 12L:12D). Ten Petri dishes were incubated per replicate, with a total of 3 replicates. Controls consisted of 10 batches of eggs laid by individual flies that were exposed to clean chambers and incubated. This was also replicated thrice. The batches were completely randomized in the incubator. Data was collected for eggs that were laid on day 1, 2, 3 and 4. T-test was used to compare means in two the two groups.

3.4.5.2 Procedure for bioassay on fertility

All the egg batches laid by flies in control and treatment were placed onto 5 x 5 cm dampened black cotton, lined in a Petri dish and incubated at a room temperatures of 26 ± 2 °C and a photoperiod of 12L:12D. Three replicates of 10 batches were observed from each group. After 96 hours, egg hatchability was assessed and recorded. The total number of open egg shells from every batch of eggs on Petri dish represented hatchability, while egg shells which remained intact after 96 hours were considered un-hatched.

Plate 3.6: Mango dome serving as an oviposition substrate

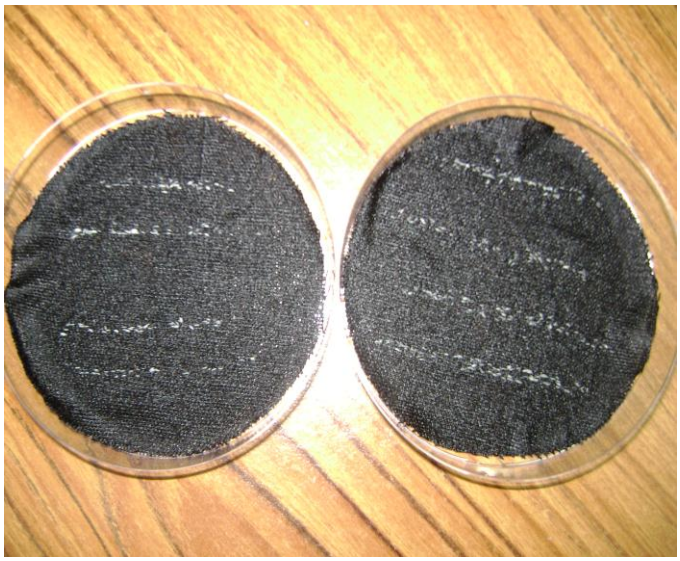


Plate 3.7: Eggs (in white) of *B. invadens* on black cotton cloth

3.5 Field Site

The field site for this study was located within ICIPE-Nairobi on a plot measuring one acre, planted with 12 mango trees of Carabao variety (Origin, Phillipines). The site is located at an altitude of 1600 meters above sea level, latitude S 01.22 and longitude E 36.90 and (GPS 315, Magellan, Taiwan) (Plate 3.10

3.6 Weather Data

Data on weather under the mango canopy was recorded during the experimental period using a data logger (Hobo and Onset Computer Corporation, USA). The data logger was placed at a height of 1 meter above the ground under mango canopy to record mean hourly temperature, relative humidity and daily precipitation of dew at the site throughout the experimental period.

3.7 Mass Production of fungus

Mature dry conidia of *M. anisopliae*, isolate ICIPE 20 was mass produced through a standard two-stage (diphasic) mass production batch system according to Jenkins *et al.,* (1998). A loop full of conidia from clean culture plate was inoculated into sterilized 50 ml liquid broth (yeast extract, glucose and peptone) in 250 ml-shake flasks. The culture was incubated at 26 ± 2 ºC for 72 h at 100 rpm in a shaker incubator to produce starter cultures (Plate 3.11). Liquid biomass was then transferred to par-boiled autoclaved rice (2 kilograms) in Milner plastic carrier bags with pre-soaked rice previously sterilized at 121 ^{0}C and 15 lb/inch^{2} (Milner *et al.*, 1998) and incubated at 26 ± 2 ºC for 10 days (Plate 3.12). The substrate was transferred onto clean plastic basins to dry at room temperature for one week by using a de-humidifier machine fixed in the production room (Plate 3.13).

Conidia were harvested by sifting the substrate through a 295 μm pore size sieve. Conidia were dried further in a dessicator using anhydrous silica gel (Plate 3.12) for two days until it attained relative humidity between 5 and 13% and kept in a refrigerator (0-4 ºC). Percentage viability of conidia was taken upon storage using the method in section 3.2.1.

3.8 Preparation of Food Baits and Contamination with *M. anisopliae*

Three model stations were tested: 1) Commercial bait, Nulure® at 2% (v/v) (Miller chemical and Fetilizer Co., Hanover, PA) on wooden sphere (9 cm in diameter and 0.5 cm thick), 2) locally developed liquid ICIPE bait (DuduLure®) at 10% (w/v) on the same wooden sphere as above and 3) dry form of the ICIPE bait (DuduLure®). The dry wooden spheres were soaked separately in mixtures of 2% Nulure® (v/v) and 10% DuduLure® bait (w/v) in water for a period of 4 hours. The spheres were then transferred into a bucket trap and placed in a shaded area to dry for a period of 2-3 days (Plate 3.13). The dry DuduLure® bait was made by transferring an aliquot of 50 ml of the paste form of the DuduLure into Petri dish and drying for 2-3 days into a semi-solid gel (Plate 3.13). Conidia of *M. anisopliae* ICIPE 20 measuring 0.3 grams was thoroughly spread on the dry baits using a glass rod spreader to assume homogeinity. Control consisted of dried baits contaminated with 0.3 grams of conidia kept in the refregirator at 0 - 4 °C throught the experimental period.

3.9 Assessment of conidial persistence, resident time of *B. invadens* and pathogencity of *M. anisopliae* on treated bait stations to adult *B. invadens*

3.9.1 Experimental design for persistence using a 2 x 2 factorial design

The field for trial consisted of an open plot measuring one acre, planted with 12 mango trees of Carabao variety, which were equally distributed by a minimum distance of 4 meters. Five canopies were randomly sampled from the plot. Treatment consisted of 10% DuduLure, 2% NuLure and DuduLure® contaminated with conidia of *M. anisopliae* and hang under each canopy. Treatments were replicated 5 times and were fully randomized within the canopies. Germination of conidia (variable) was observed as affected by environment and bait type (factors). Impact of the environment on conidia over time was carried out using repeated measures on days 0, 7, 14, 21 and 28. Controls consisted of the 3 bait types without fungus, kept between 0 and 4 °C . The study period ranged from 9th May 2006 to 12th June 2006.

3.9.2 Assessment of conidial persistence on contaminated bait stations

The contaminated baits prepared in section 3.8 above were hang in the interior part of the mango canopy at a height of approximately 2 metres above the ground and close to a bunch of leaves (Plate 3.15a and Plate 3.15b). Approximately 6-7 stations were hung in the tree. On day 0, 7, 14, 21 and 28 (from May 9, to June 12th, 2006) the contaminated baits were removed from the trees for viability tests. Using sterilized forceps and cotton swab, conidia from the contaminated bait was picked by swabbing and transferring conidia into universal bottle containing 1 ml of 0.01% triton X-100 and glass beads. The suspension was vortexed for 5 minutes and 0.1 ml was inoculated by spread plate method

on a Petri dish of Sabouraude Dextrose Agar. A total of 5 replicates per bait were tested for viability. The plates were incubated at temperatures of 26 ± 2 ºC for a period of 24 hours and percent germinated conidia recorded using the procedure germination count method explained in section 3.2.1.)

3.9.3 Assessment of resident time of *B. invadens* on fungus contaminated baits

Three different dry food baits (2% Nulure®, 10%DuduLure®) and DuduLure®) treated with 0.3 g of *M. anisopliae* were hang in plexi glass cages that measured 30 x 30 x 30 cm (Plate 3.14). A single fly was removed from a cohort of 1000 flies (that were previously starved for 24 h) in a clean cage using falcon tube that had one end screened and held from side of the plexi-glass that is open, approximately 12 cm away from the hang bait. Once the fly landed on the station, its resident time on the station was recorded using a stopwatch (Zyliss, Switzerland). The resident time of adult *B. invadens* to the three different baits treated with fungus was recorded. A total of five spheres of fungus contaminated food baits were hanged per bait. Controls consisted of the three different baits without fungus.

3.9.4 Experimental Design for pathogenicity a using single factorial arrangement

The environmental impact on *M. anisopliae* contaminated baits on populations of adult *B. invadens* was evaluated in a completely randomized design experiment. Treatment consisted of 10% DuduLure, 2% NuLure and DuduLure® contaminated with conidia of *M. anisopliae* and , replicated 5 times. Treatment replications were fully randomized within the experimental room. The experimental units was a replica of 10 flies receiving

infection from each bait type, replicated 5 times with mortality as the variable and period of exposure as a factor. Impact of environmental exposure on treated baits to *B. invadens* population over time was carried out using repeated measures on days 0, 7, 14, 21, 28. Controls consisted of baits without fungus. The study period was 9th May 2006 to 12th June 2006.

3.9.5 Assessment of Pathogenicity of *M. anisopliae* in bait stations to adult *B. invadens*

The infection process in this section was similar to the procedure explained in section 3.9.3 for resident time. The stations were tested by hanging them individually at the top of Plexiglas cages measuring 30 x 30 x 30 cm in the laboratory. Five stations (replicates) from each treatment were hang. A single fly was removed from a cohort of 1000 flies (previously starved for 24 h) in a clean cage using falcon tube that had one end screened. The open end of the tube was then placed approximately 12 cm away from the bait. Individual flies which took 30 seconds or more to pass through the contamination device were captured using aspirator upon exit and transferred and maintained individually into special falcon tubes which had one end screened and a mixture of sugar and enzymatic yeast hydrolysate and water on pumice granules as a food. Five replicates of 10 insects per treatment were maintained at temperatures of 26 ± 2 ºC and relative humidity of 85%. Mortality was observed and recorded daily for a period of 5 days. Dead flies were surface sterilized for mycosis using procedure in Section 3.2.3. Controls consisted of flies visiting untreated-baiting stations. All tests were conducted on day 0, 7, 14, 21 and 28 days post exposure.

Plate 3.8: Field site, 1600 meters above sea level; 01.2 S, 36.9 E showing mango trees used during persistence experiment, ICIPE, Nairobi

Plate 3.9: Starter cultures of *M. anisopliae* in orbital shaker incubator

Plate 3.10: Mass production of *M. anisopliae* using rice in Milner bags

Plate 3.11: Mass produced conidia of *M. anisioplaie* undergoing dehydration and aeration at room temperature

Plate 3.12: Desiccation of conidia of *M. anisopliae* using silica gel

Plate 3.13: Baits hang to dry under shade

Plate 3.14: Dry baits treated with spores of *M .anisopliae* used for infection

Plate 3.15 a: Spheres of Baits contaminated with *M. anisopliae* and placed under mango canopy

Plate 3.15 b: *Metarhizium anisopliae*-treated bait under mango canopy

3.10 Data analysis

3.10.1 Analysis of Pathogenicity of *M. anisopliae* and *B. bassiana* isolates to adult *B invadens*

Mean percent mortality and number of conidia picked per fly was also determined using one -way ANOVA. Mortality data were corrected for natural mortality using Abbott's formula (Abbott, 1925) then normalized by arcsine transformation. One way ANOVA was used to analyze mean percentage mortality using PROC GLM procedure of SAS (SAS, 2001). The means were separated using Tukey's studentized range test (1953) (α = 0.05). The lethal time to kill 50% (LT_{50}) were determined using probit analysis procedure of SAS (Throne *et al*., 1995).

3.10.2 Analysis of fecundity and fertility

Mean- fecundity and fertility per day, gross fecundity and fertility by individual females during the experimental period were recorded for each replicate and analyzed using paired t-test for comparison of means (Gossett, 1908) after first checking the data for an approximation to a normal distribution by ln (x + 1). Percentage reduction in fertility and fecundity were calculated according to Castillo *et al*., (2000):

$$\text{Percentage reduction} = \frac{\text{(Number of eggs in control - number of eggs in treatment)}}{\text{Number of eggs in control}} \times 100$$

/

3.12.3 Analysis of Response time

The data on resident time is demonstrated in a frequency distribution table using class intervals of seven seconds and show the resident time spent by flies on each bait.

Kurskal-Wallis Rank test for non-parametrics was used to test compatibility of adult *B. invadens* to the three baits:

$$H = \frac{12}{N}(N+1)\frac{(\Sigma R^2)}{n} - 3(N+1):$$

where n = sample variation; R= rank totals of each sample; 3 and 12 = constant; Σ = total observations.

3.12.4 Data analysis of Conidial persistence of conidia over time and pathogenicity

One-way analysis of variance was used to analyze percentage conidial germination (survival) and mortality data (at five days post infection) using PROC GLM procedure of SAS after arcsine transformation to normalize the data. Before the analysis was carried out, conidial survival was estimated by comparing the viability of irradiated conidia with the viability of the control conidia (Goettel and Inglis, 1997):

$$\text{Percentage survival} = \frac{\text{number of viable conidia following irradiation}}{\text{number of viable conidia in control}} \times 100$$

Data on fly mortality was corrected using Abbott's formula of correction (Abbott, 1925). Interaction between time and treated baits was analyzed using two-way analysis of variance for a randomized complete block design with replicates. Means were separated using Tukey's test (P = 0.05).

CHAPTER 4

4.0 RESULTS

4.1 Laboratory Bioassays on Pathogenicity of *M. anisopliae* and *B. bassiana* isolates

4.1.1 Assessment of Pathogenicity between the 2 isolates based on exposure time

Mortality was not significantly different between the two *M. anisopliae*-treated flies when exposed to conidia of ICIPE 20 and ICIPE 62 at different time intervals throughout the experimental period, although the mean number of conidia picked per fly varied between the isolates and ranged from 1×10^4 to 1.29×10^6 conidia (Table 4.1). The number of conidia picked per fly increased with exposure time in both isolates. Flies spent shortest time of 30 seconds on conidia-treated chamber of ICIPE 20 (lowest dose) demonstrated shorter LT_{50} than those exposed for 3 minutes on the same chamber. Comparative results between the 2 isolates at 30 seconds of exposure showed LT_{50} of 3 days and a lower dose of *Metarhizium* conidia picked in isolate ICIPE 20 (1×10^4) than ICIPE 62 (2×10^5) (Table 4.1). When flies were exposed for 3 minutes, flies from ICIPE 20-treated chamber picked an average of 1.29×10^6 conidia/fly, which is 5 times higher than conidia picked by flies exposed to isolate ICIPE 62 treated chamber (2.75×10^5) and had LT_{50} of 3.1 days and 2.8 days respectively.

Table 4.1: LT_{50} response, number of conidia picked/fly and mean mortality at 5 days post infection of adult *B. invadens* exposed to *M. anisopliae* ICIPE 20 and 62.

Fungi	Exposure period	conidia/fly	mean %-age mortality ± SE	LT_{50} days	Chi-square
ICIPE 20	30secs	$1x10^{4}$	100 ± 0.0a	3.0	526.23
	1min	$7.4x10^{4}$	95.6 ± 4.4a	2.7	520.57
	2 min	$5.27x10^{5}$	95.6 ±4.4a	2.8	526.47
	3 min	$1.29x10^{6}$	96.7 ± 1.3a	3.1	535.69
ICIPE62	30secs	$2x10^{5}$	100 ±0.0a	3.0	522.78
	1min	***	100 ± 0.0a	3.0	527.18
	2 min	$3.3x10^{5}$	100 ± 0.0a	3.5	525.75
	3 min	$2.75x10^{5}$	100 ± 0.0a	2.8	526.23

*** missing data

4.1.2 Evaluation of most pathogenic isolates of *M. anisopliae* and *B. bassiana*

In viability test, percentage germination of conidia for all isolates ranged from 79.2-96.8% after 16 hours of incubation (Table 4.2). Out of 24 isolates tested, 16 isolates (ICIPE 18, 7, 95, 20, 58, 62, 49, 53, 69, 21, 387, 402, 295, 282, 603 and 273) exhibited significantly higher percentage germination than the others ($F=12.24$; df = 21, 84; $P< 0.0001$) (Table 4.2). In the pathogenicity test (Table 4.3), mortality in the control treatment was 4.0% at 5 days post treatment. All fungal treated flies remained active for the first two days, but subsequently started showing signs of infection. Mortality among the 24 isolates ranged from 18.7 to 94.8%. Results show that mortality was significantly higher in five isolates of *M. anisopliae* (ICIPE 62, 20, 43, 69, 23) and two isolates of *B. bassiana* (ICIPE 295 and 303) than the rest of the isolates ($F= 21.86$; df = 21, 84; $P<0.0001$) (Table 4.3); and were therefore considered most pathogenic at 5 days post treatment. Lethal time to kill 50% of the flies (LT_{50}) for these isolates ranged from 2.8 to 3.6 days (*M. anisopliae*) and 2.70 to 2.78 days *(B. bassiana*) (Table 4.3). Results show higher slope value estimates of 0.96 for 2 isolates of *M. anisoapliae* (ICIPE 20, 62) and slope of 0.88 for *M. anisopliae* ICIPE 52, 53, and *M. anisopliae var. acridium* ICIPE 23. Two isolates of *B. bassiana* (ICIPE 295, 303) had the highest slope value estimates of 0.88 each (Table 4.3).

Table 4.2: Percentage viability of the different fungal isolates on SDA plates incubated at 26 °C for 16-18 h.

Fungal species	Accession number	% germination ±SE
Metarhizium anisopliae	ICIPE 18	96.0 ± 1.1a
	ICIPE 55	88.6 ±1.9 bc
	ICIPE 41	79.2 ±1.8 d
	ICIPE 7	90.0 ± 2.7 abc
	CIPE 95	92.2 ±1.2 abc
	ICIPE 20	96.0 ±1.2 a
	ICIPE 58	94.6 ±1.4 ab
	ICIPE 62	91.6 ±1.6 abc
	ICIPE60	89.0 ±2.3 cb
	ICIPE 49	96.8 ± 0.8 a
	ICIPE53	94.2 ±1.2 ab
	ICIPE 69	96.8 ±0.8 a
	ICIPE 23	90.6 ±1.5 abc
	ICIPE 387	96.0 ± 1.0 a
	ICIPE 402	90.2 ± 2.1 abc
Beauveria bassiana	ICIPE 33	87.43 ± 1.4 bc
	ICIPE 43	88.8 ±1.6 bc
	ICIPE 295	91.0 ±1.1 abc
	ICIPE284	83.0 ±1.7 cde
	ICIPE282	93.4 ±2.9 ab
	ICIPE 280	87.42 ± bc
	ICIPE 303	87.2 ±1.8 bcd
	ICIPE 603	94.2 ± 1.1 ab
	ICIPE 273	91.2 ± 1.9 abc

Table 4.3: Mean percentage mortalities and LT_{50} values of f adult *Bactrocera invadens* infected with *Metarhizium anisopliae* and *Beauveria bassiana* isolates at 5 days post infection.

Fungal Isolates	Accession number	mortality ±se	LT_{50}(days) (95% CL)	slope	χ^2
M. anisopliae					
	ICIPE 18	46.73 ± 7.85def	6.70 (6.0-7.3)	0.39±0.13	377.04
	ICIPE 55	64.49 ± 9.73 abcde	4.74 (4.6-5.0)	0.58±0.14	420.14
	ICIPE 95	88.55 ± 12.09ab	6.0 (5.5-6.7)	0.38±0.13	399.95
	ICIPE 7	29.2 ± 7.56 efg	11.65 (9.33-16.14)	0.24±0.12	351.49
	ICIPE 62	93.73± 1.06 a	3.09 (3.02-3.15)	0.96±0.14	534.47
	ICIPE 20	94.77 ± 0.20 a	3.08 (3.01-3.14)	0.96 ± 0.14	534.44
	ICIPE 49	87.46 ± 4.57 ab	3.61 (3.54-3.69)	0.71 ± 0.13	527.10
	ICIPE 60	86.51 ± 5.31 ab	3.15 (3.02-3.28)	0.77±0.13	411.15
	ICIPE 69	94.79 ± 0.02 a	3.39 (3.31-3.47)	0.88±0.13	528.68
	ICIPE 43	94.79 ± 0.02 a	3.42 (3.35-3.50)	0.88±0.14	526.94
	ICIPE 53	88.55 ± 0.02 ab	3.53 (3.44-3.61)	0.90±0.14	518.84
	ICIPE 41	45.81 ± 4.81def	6.96 (6.00-8.50)	0.23±0.11	324.91
	ICIPE 58	90.64 ± 2.99 ab	3.29 (3.21-3.36)	0.91±0.14	324.91

Continuation of **Table 4.3**

	ICIPE 387	47 ± 11.2 def	5.3 (5.2 - 5.5)	0.40±0.09	552.17
	ICIPE 402	20.00± 5.7 fg	12.8 0 (11.1-15.1)	0.09±0.08	479
M. anisoplaie var. *acridium*					
	ICIPE 23 (IMI 330 189)	94.79 ± 0.02a	3.39 (3.31-3.47)	0.88±0.14	528.68
B. bassiana	ICIPE 33	67.79 ± 12.15 abcde	4.64 (4.43-4.90)	0.52±0.13	436.28
	ICIPE 284	64.60 ± 4.45 abcde	5.0 (4.7-5.3)	0.48±0.12	423.93
	ICIPE 280	69.76 ± 8.32 abcd	4.4 (4.2-4.5)	0.60±0.13	445.77
	ICIPE 282	18.67 ± 4.15 fg	72.8 (29.6-548.09)	0.224±0.06	318.26
	ICIPE 303	94.79 ± 0.02a	2.78 (2.70-2.84)	0.88±0.12	522.57
	ICIPE 295	94.79± 0.02 a	2.70 (2.6-2.8)	0.81±0.12	507.82
	ICIPE 603	30.00 ± 8.3	6.70 (6.4-6.9)	0.29±0.08	762
	ICIPE 273	75.64 ± 5.7	3.9 (3.7-4.0)	0.42±0.08	536

Means ± SE for each parameter within a column followed by the same letter are not significantly different by Turkey's test (P = 0.05).

4.1.3 Estimation of mean number of conidia picked per fly

Mean number of conidia picked per fly exposed for 3 minutes varied from 6.4 x 10^5 to 1.29 x 10^6 among 4 isolates of *M. anisopliae* (ICIPE 41, 18, 7, 55) (Table 4.4). Results showed that significantly higher number of conidia were picked/fly in ICIPE 55 than ICIPE 7. (Table 4.4).

Table 4.4 Mean number of conidia picked per fly after 3 minutes exposure of isolates of *Metarhizium anisopliae.*

Fungal Species	Fungal Isolate	Mean no. of propagules (ml^{-1})
M. anisopliae	ICIPE 41	8.4 x10^5 ± 0.6 ab
	ICIPE 18	1.29 x10^6 ± 1.09 ab
	ICIPE 55	1.70 x10^6 ± 5.2 a
	ICIPE 7	6.4 x10^5± 0.8 b

Means ±SE for each parameter within a column followed by the same letter are not significantly different, by Turkey's test. P = 0.05.

Plate 3.16: Adult *Bactrocera invadens* mycosed with *Beauveria bassiana*

Plate 3.17: Adult *Bactrocera invadens* mycosed with *M. anisopliae*

4.2 Laboratory Bioassays on Fecundity and Fertility

This bioassay tested viability and effect of *M. anisopliae* isolate ICIPE 20 on fecundity and fertility of adult *B. invadens*. In viability tests, germination of *M. anisopliae* isolate ICIPE 20 ranged from 94-96%. In general, the isolate was found to cause significant sub-lethal effects on egg fecundity and fertility when gravid female *B. invadens* were infected with the fungus.

4.2.1 Assessment of Fecundity

Mean daily egg production over the 4-day experimental period in the control was 28.4 eggs/fly/day and 6.8 eggs /fly/day in the treated flies (Table 4.5). The gross data stood at 113.2 eggs/female over 4 days in the control against those in treated flies at 28.8 eggs/ female over 4 days post inoculation (Table 4.5). Egg fecundity was not significantly different on day 1 between fungus treated flies (21.2 eggs) and controls (17.1 eggs) (t_{18} = 0.458 $P < 0.01$) (Figure 4.1). However fecundity was significantly lower in fungus-treated flies (4.1 eggs) than in control (20.8 eggs) on day 2 (t_{18} = 2.69; P = 0.05) with a comparative reduction of 80.2% (Figure 4.1). Egg fecundity was also significantly lower in treated flies than controls on days 3 and 4; treated flies laid 1.6 and 0.1 against control at 19.4 and 49.9 eggs, respectively (t_{18} = 2.98; P < 0.01) and (t_{18} = 5.28; P = < 0.01) (Figure 4.1) and exhibited reductions of 91.9% and 99.8% (Figure 4.1).

Table 4.5: Mean ± SE fecundity, cumulative fecundity and hatchability per fly per day over 4 days post inoculation in *Bactrocera invadens* infected with *Metarhizium anisopliae* ICIPE 20 in the laboratory

Treatment	Number of eggs laid/female/day	Cumulative fecundity/ female	Cumulative hatch rate/female/day
Control	28.4 ± 1.0 a	113.2 ± 5.0 a	64.8 ± 8.9
M. anisopliae	6.8 ± 1.2 b	28.8 ± 5.2 b	17.2 ± 9.3

Means ±SE for each parameter within a column followed by the same letter are not significantly different, P = 0.05.

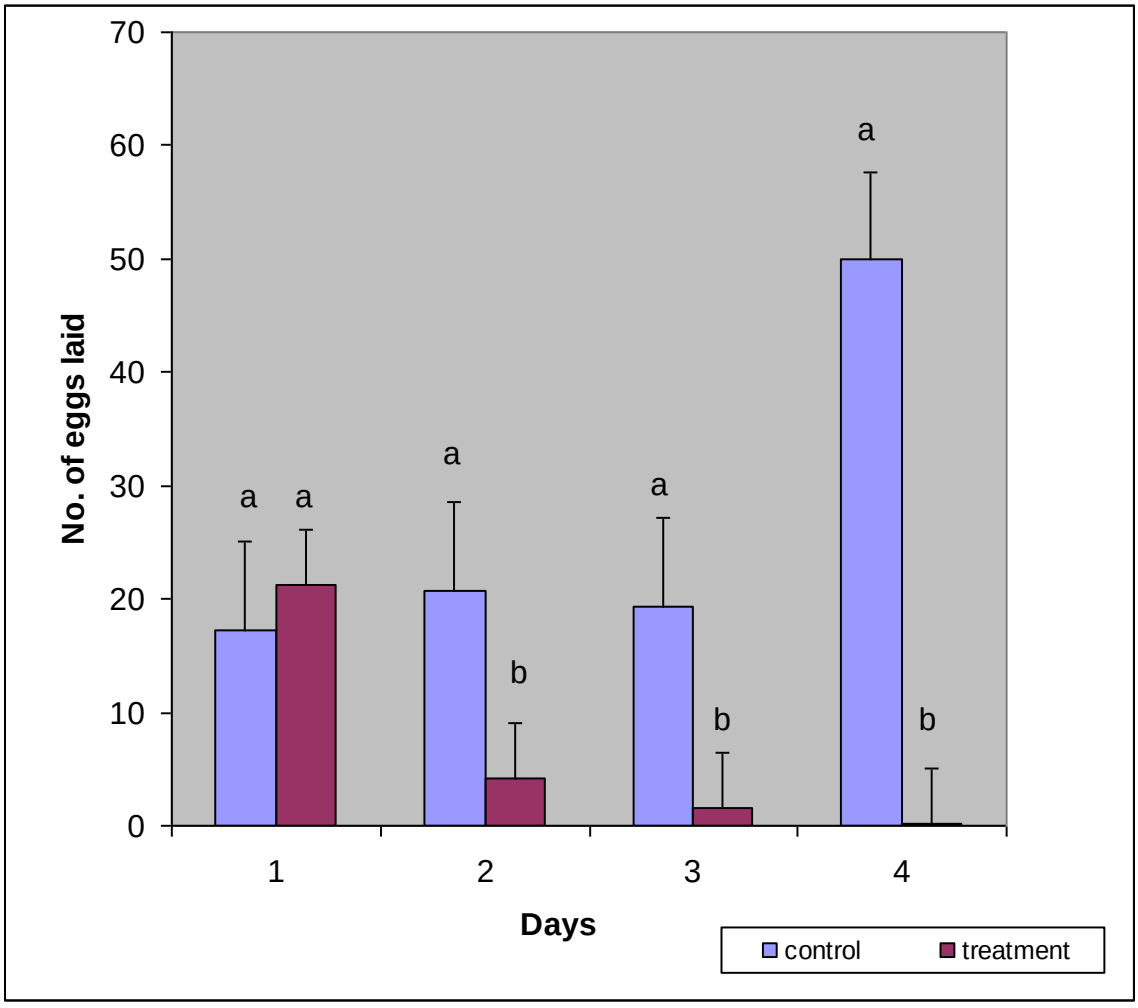


Figure 4.1: Mean number of eggs per day laid by *B. invadens* infected with *M. anisopliae*

4.2.2 Assessment of Fertility

The overall results demonstrated that hatchability of eggs was significantly lower in fungus treated flies than in control (Figure 4.2). Gross hatchability of eggs laid by flies in control was 64.8% and 17.2% in treated flies respectively (Figure 4.2). When effect of the fungus on subsequent egg hatchability was analyzed and compared with those in controls, day 1 results showed that hatchability was significantly higher in eggs that were laid by flies from control than treated flies (t_{13} =1.148; P = 0.05) (Figure 4.2). However on day 2, hatchability was significantly lower in eggs laid by fungus-treated flies (24.3 %) than controls (49.9%) (t_{10} = 2.083; P = 0.05) (Figure 4.2). This amounted to a reduction in hatchability of 51.3%. On day 3, egg hatchability was significantly lower in eggs laid from fungus treated flies (15.1%) than in control (76.4 %) (t_{10} = 3.57; P < 0.05); and a reduction of 80.2% in hatchability compared to control (Figure 4.2). On day 4, no eggs hatched from fungus treated resulting into a significantly lower (0%) hatchability in eggs than in control (76.4 %) (t_9 = 10.47; P < 0.01).

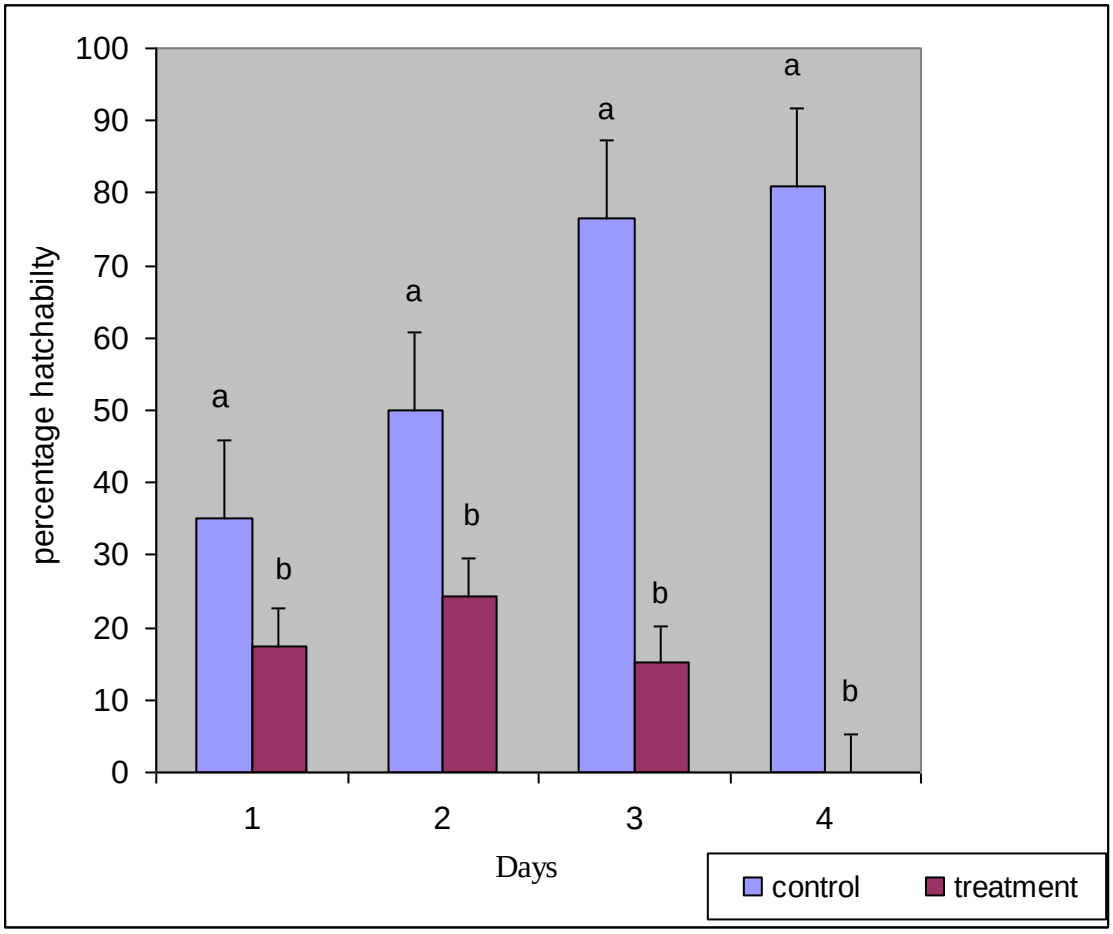


Figure: 4.2: Mean percentage hatchability of eggs laid by *B. invadens* infected with *M. anisopliae*

4.2.3 Pathogenicity of *M. anisopliae* ICIPE 20 on gravid females of *B. invadens*

In pathogenicity, no mortality occurred in fungus treated insects and in control on days 1 and 2 post inoculation. However, mortality was significantly higher in fungus-treated flies than in controls (3.3%) on day 3 and 4 and 93.3% and 100% in treated (t_{18} =34.96; P < 0.01) and 3.3% in control (t_{18} = 53.13; P < 0.01) post inoculation (Table 4.6). All fungus treated flies died by 4 days post inoculation.

Table 4:6: Mean ± SE daily percentage mortality of 10-14 days old gravid females infected with *M. anisopliae* in the laboratory.

Treatment	Days post infection			
	1	2	3	4
Control	0.0 ± 0.0 a	3.3 ± 1.8 a	3.3 ± 1.8 b	3.3 ± 0.1.8 b
M. anisopliae	0.0 ± 0.0 a	3.3 ± 1.8 a	93.3 ±1.8 a	100.0 ± 0.0 a

Mean ± SE for each parameter within a column followed by the same letter are not significantly different, P = 0.05.

4.3 Assessment of persistence and subsequent pathogenicity of *M. anisopliae* exposed in the field and residence time of *B. invadens* on contaminated baits

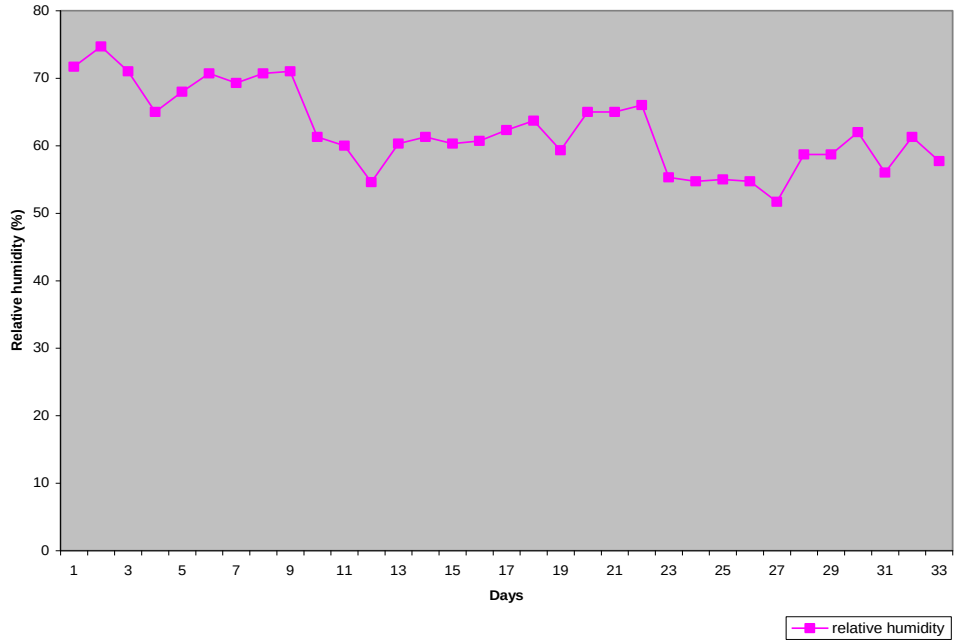
4.3.1 Weather results

Maximum and minimum temperature and relative humidity under the mango canopies in the field fluctuated from 17.3 to 24.1 °C and 53 % and 76% respectively throughout the experimental period 9th May to 12th June 2006 (Figure 4.3).

4.3.2 Assessment of residence time to fungal contaminated bait stations

After flies were released into the cages, results showed that over 80% of the flies spent between 1 and 28 seconds on the contamination device upon landing on both treated and control baits. Resident time of flies was not significantly different on the three *M. anisopliae*-treated baits (Nulure®,10%DuduLure and Dry DuduLure) that were 1 and 35 day old baits ($\chi^2_{3\text{-}1} = 1.198$; $P > 0.05$), ($\chi^2_{3\text{-}1} = 0.4$; $P > 0.05$). In the controls, resident time of the flies was also not significantly different among the 3 baits ($\chi^2_{3\text{-}1} = 0.484$; $P > 0.05$). It was not possible to compare data for 35-day old baits in control because of heavy environmental contaminant on dry DuduLure® bait (Table 4. 7a and b).

A



B

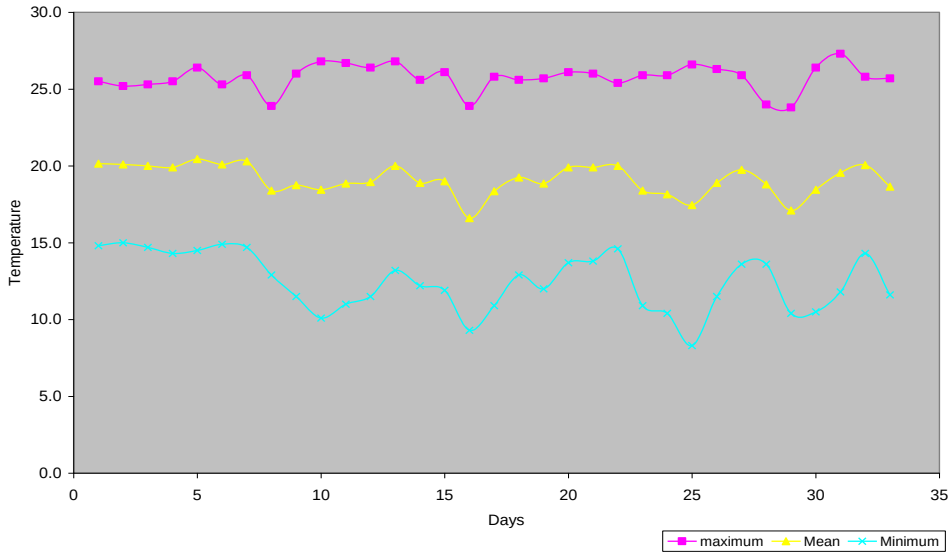


Figure 4.3: Overall pattern of temperature (A), relative humidity (B) under mango canopy in the field where treated baits were exposed

Table 4.7a: Residence time of adult *B. invadens* on fresh bait stations contaminated with *M. anisopliae* conidia in the laboratory.

	No of flies landed on bait station					
	Metarhizium anisopliae			Control		
Residence time (seconds)	2% Nulure +fungus	10% DuduLure + fungus	DuduLure® + fungus	2% Nulure	10 %Dudu-Lure	Dudu Lure®
1-7	39	43	27	13	18	23
8-14	5	4	5	13	6	3
15-21	2	0	1	5	4	7
22-28	0	0	3	2	3	0
28-35	0	1	0	3	3	1
36-42	0	0	2	0	0	0
43-49	0	0	0	0	0	0
50-56	0	1	1	3	1	0
57-180	4	1	10	10	18	16

Table 4.7b: Residence time of adult *B. invadens* on 28 day old *M. anisopliae* contaminated bait stations .

	No of flies landed on bait station					
	Metarhizium anisopliae			Control		
Residence time(second interval)	2%Nulure +fungus	10% DuduLure + fungus	DuduLure® + fungus	2% Nulure	10%Dudu-Lure	Dudu Lure®
1-7	41	34	31	21	33	-
8-14	1	5	9	13	8	-
15-21	2	2	3	3	2	-
22-28	1	1	3	1	1	-
28-35	0	0	0	1	0	-
36-42	0	0	0	0	1	-
43-49	0	1	0	0	0	-
50-56	0	0	0	1	0	-
57-3min	3	6	4	10	4	-

4.3.3 Assessment of conidial Viability on Food baits over time

Overall, the rate of reduction in viability over time was significantly higher in conidia spread on the dry DuduLure® bait station compared to the fungus treated- 2% NuLure and 10% DuduLure bait (Table 4.8). Results from one- way analysis of variance exhibited that there was no significant effect of any of the 3 baits on germination of conidia. Viability of conidia on day 7 post field exposure was not significantly different among the 3 baits and was at 93.1% in the 10% DuduLure, 85.5% in 2% NuLure) and 100% in the dry DuduLure® stations (F = 264.03; df, 2,15; P = 0.0001) (Table 4.8). However, on day 14 post exposure conidial viability was significantly different among all the 3 bait stations, with 10% DuduLure) exhibiting significantly higher percentage germination of 83%, than on 2%NuLure (74%) and DuduLure®) (53.6%) (F = 100.21; df = 2, 15; P = 0.0001). On day 21, conidial viability in 10% DuduLure (94.6%) bait and 2%Nulure (96%) was significantly higher than in DuduLure®) (6.1%), however conidial viability was not significantly different between the 2 baits (10% DuduLure and 2% Nulure) (F = 515.82; df = 2, 14; P = 0.001) (Table 4.8). Viability was significantly different in all the 3 baits at 28 days of exposure; 2% NuLure exhibited significantly higher conidial germination (96.3%) than 10% DuduLure (78.2%) and DuduLure® (6.5%) (F = 298; df 2, 14; P = 0.001). A high population of *Rhizopus* had colonized AFFI solid baits that were collected from the field on day 21 of exposure. Results from two-way analysis of variance exhibited that there was no significant effect of any of the 3 baits on germination of conidia (F= 0.107; df = 2, 75; P = 0.05); but there was significant effect of time on germination of conidia (F = 175; df= 4, 75; P = 0.005) (Table 4.8). Results also demonstrated that there was significant effect due to interaction between

treated baits and time (F= 74.6; df = 8, 75; P = 0.005) in viability. A gradual decrease in conidial viability of *M. anisopliae* was observed in all the 3 baits between day 1 and 28. Over this period, viability ranged from 100% to 96% in 2% NuLure®, 100% to 6.5% in the dry DuduLure bait station; and 100% to 78% in 10% DuduLure bait (Table 4.8).

Table 4.8: Comparison of viability of *Metarhizium anisopliae* (ICIPE20) on three different baits exposed for a period of 28 days field under mango canopy.

Treatment	Day 0	Day 7	Day 14	Day 21	Day 28
Dry DuduLure®	100 ± 0.0aA	100 ± 0.0 aA	53.6 ± 0.8 cAB	6.1 ± 0.0 bB	6.5 ± 1.4 cB
10% DuduLure	100 ± 0.0aA	93.1 ± 1.1bA	83.0 ± 1.4 aA	96.0 ± 1.6a A	78.2 ± 3.3 bA
2% NuLure	100 ± 0.0aA	85.5 ± 0.3 cA	74.0 ± 2.4 bA	94.7 ± 1.5 aA	96.3 ± 2.6 aA

Column means of germination (lower case) or row means of germination (in upper case) (± SE) bearing the same letter are not significantly different by Turkey's test (P = 0.05).

4.3.4 **Evaluation of Pathogenicity of *M. anisopliae* on bait stations to adult *B. invadens***

Pathogenicity results showed a decreasing trend of mortality of *B. invadens* in all baits over time. On day 0, mortality of flies was significantly higher from fungus treated 10% DuduLure (98%) and 2% Nulure (100%) baits than in DuduLure®) (95%), however mortality was not significantly different between flies infected from 10% DuduLure and 2% Nulure ($F = 10.76$; df 2,12; $P < 0.05$) (Figure 4.4). On day 7 mortality was not significantly different between flies exposed to fungus treated 2% Nulure (100%) and 10% DuduLure (100%); however the two mortalities were significantly higher than mortality from DuduLure®) (84%) ($F = 10.76$; df = 2, 12; $P < 0.05$) (Figure 4.4). On day 14, mortality was not significantly different between flies infected from fungus treated-10% DuduLure (95%) and 2% Nulure (84%) baits; but these mortalities again differed significantly from mortality in fungus treated DuduLure® (50%) bait ($F = 6.10$; df 2,12; $P = 0.05$) (Figure 4.4). A similar trend was exhibited on day 21 with no significant difference in mortality between flies infected from 10% DuduLure (83.5%) and 2% NuLure (64.0%) baits; but these mortalities were significantly higher in the two treatments and mortality in fungus-treated DuduLure® (5.7%) ($F = 32.67$; df 2,12; $P = 0.05$) (Figure 4.4). Due to heavy contamination by mould on dry DuduLure® by day 28, bioassay on its pathogenic effect was not carried out (Figure 4.4) except on 2 % NuLure and 10% DuduLure. Comparison of means using paired t-test demonstrated mortality was significantly higher in 2% NuLure than in 10%DuduLure® and measured 89.8% and 47.6% respectively ($t_{10} = 15.4025$; $P = 0.05$).

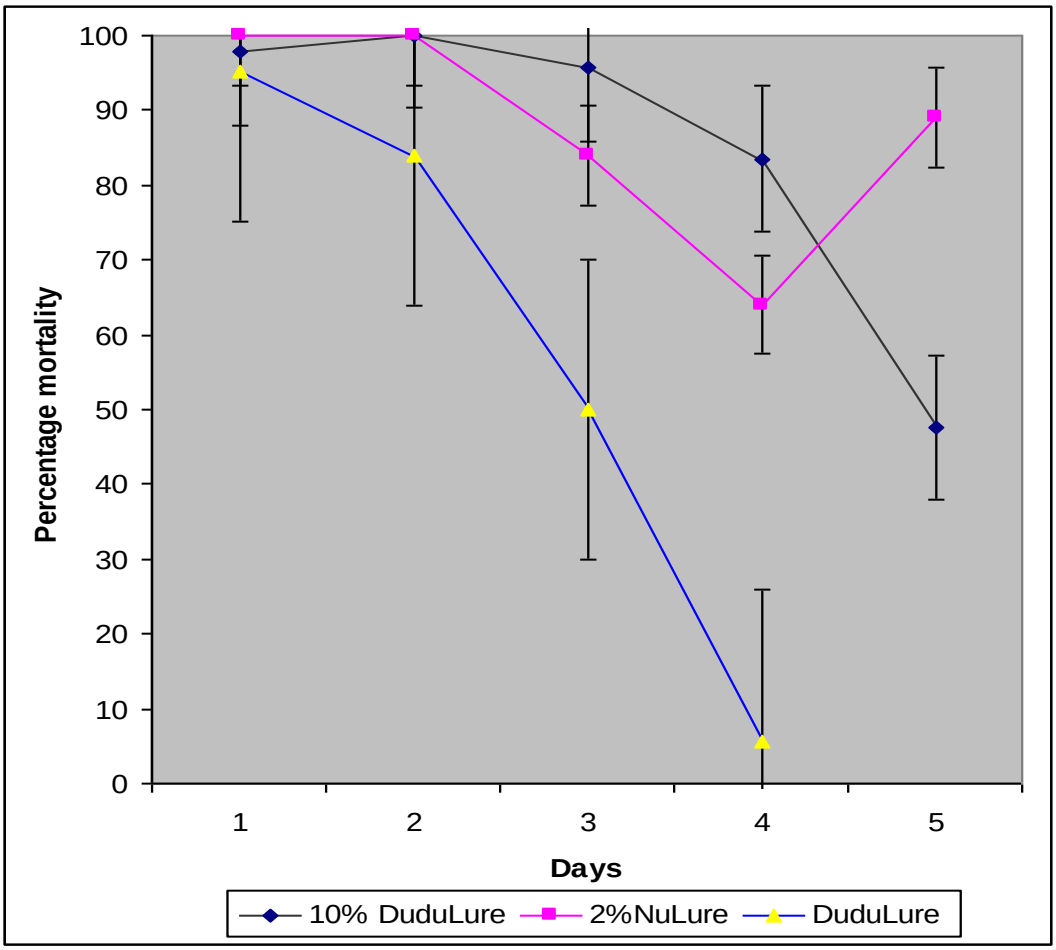


Figure: 4.4: Mean cumulative mortality of adult *B. invadens* following exposure to bait stations contaminated withy *M. anisopliae* .

CHAPTER 5

5.0 DISCUSSION

Initial bioassay that assessed the effect of exposure duration on fly mortality showed that no significant difference in mortality occurred among the 4 durations of exposure tested regardless of the number of conidia picked per fly (Table 4.1). As a result, subsequent screenings were done at the lowest exposure time of 30 seconds. The fact that low conidial dosage of 1 x 10^4 picked up within 30 seconds of exposure was able to induce 100% mortality in adult flies suggest that a prolonged period of time on baiting stations with fungal spores may not be needed to induce lethal levels of mortalities on adult *B. invadens*.

In the present study, no fungal hypha was observed outside the host integument before its death and even then, the fungus formed surface mycelia only after incubation at high humidity. Visible mycelial growth could be observed at the inter-segmental membrane of the abdomen, mouth parts, tibia pads and neck at 48 h after death of the insect. At five days after incubation, profuse sporulation was observed on mummified adults. A high level of sporulation on the surface of the cadaver should be an important criterion for inoculum dispersal in the field because each cadaver constitutes an infection focus. Infection foci may play an important role in the control of *B. invadens* through secondary infection, long-term persistence and overall epizootiology of the disease within mango agro-ecosystem as demonstrated by Ekesi *et al*. (2002) and Mochi *et al*. (2006) on experiments with adult *C. capitata* and pupariating larvae.

All the fungal isolates used in this study were pathogenic to adult *B. invadens* when the insects were exposed to dry conidia of *M. anisopliae* and *B. bassiana* in inoculating chambers. Generally *B. invadens* survived the first 2 days following fungal application but afterwards, a progressive fly mortality was observed. Out of the 24 isolates screened for pathogenicity against *B. invadens*, 7 isolates (ICIPE 20, 43, 62, 69, 295, 303 and *M. anisopliae var. acridium* IMI 330189 or ICIPE 21, available as Green Muscle®) induced the highest level of mortalities. Considerable variation in pathogenicity both between species and within species was observed during the bioassay. Similar variations in pathogenicity among isolates against other species of fruit flies were previously reported in *M. anisoplaie* and *B. bassiana* against *C. capitata* (Dimbi *et al*., 2003; Castillo *et al*., 2000; Garcia *et al*., 1984; Moraga *et al*., 2006). Both species of entomopathogenic fungi tested in this study have wide geographic distribution (Hall and Papierok, 1982). These isolates also generally had smaller LT_{50} values and higher slope gradient compared with the least pathogenic isolates. Among these 7 isolates, dry conidia of *M. anisopliae* ICIPE 20, 62 and 69 have been previously reported to be highly pathogenic to several tephritid species causing mortalities of 86.7% in *C. capitata,* 94.9% in *C. fasciventris* and 72.1% in *C. cosyra* at 27-28 °C (Dimbi *et al*., 2003), which is within the temperature range evaluated in this bioassay. Because *Bactrocera invadens* also occupy the same ecological niche on mango with the tephritid species listed above (Lux *et al*., 2003; Ekesi *et al*., 2006), any of the three isolates would therefore be an ideal candidate against the fruits flies in mango agrocosystem. Konstantopoulou *et al*. (2005) tested liquid suspension by feeding adult *C. capitata* on $1x10^4$ conidia ml^{-1} in the laboratory and recorded mortality of 85.6% and 97.4% by *B. bassiana* and *B. brongniartii* but the same isolates were less

pathogenic on adult *Bactrocera olae* Gmelin achieving 62.6% and 36.5% mortality at 21 days post infection. Toxicity results on oral dosing of adults of *C. capitata* using extracts from *B. bassiana* at concentration of 25mg/g of diet resulted into 90% mortality (Castillo *et. al.* 2000). By applying conidial suspension, these authors probably envisaged foliar spray of liquid suspension but this would seem economically questionable for tree crops given that large volume of suspension and labour would be required. The use of dry conidia as tested in the last experiment of this study in autodessimination devices may perhaps be the most viable control method in mango orchards.

The best results obtained with entomopathogenic fungi by most authors are with isolates obtained from the test insects or from closely related species (Zimmerman, 1982; Poprawski *et al.*, 1985; Feng and Johnson, 1990; Ekesi *et al*., 2000). However, in this study, the 7 candidate isolates came from different sources and or substrates other than fruit flies. Other isolates that were obtained during pathogen survey of this study around mango growing regions such as *M. anisopliae* (ICIPE 402, 387) and *Beauveria bassiana* (ICIPE 603) were less pathogenic. These results suggest that although widely distributed fungi such as *B. bassiana* and *M. anisopliae* could be relatively host-specific as pathotypes, isolates from other host and or environment such as soil also offers potential for high virulence and warrant exploration.

Among the 7 most pathogenic isolates, *M. anisopliae var. acridium* IMI 330189®, which registered product for the control of locust and grasshoppers (Milner *et al.*, 1998) also displayed high levels of pathogenicity to adult *B. invadens.* Since the isolate exhibits

broad-spectrum activity against Acrididae (Orthoptera) and some members of Tephritidae (Diptera) (Milner *et al.,* 1998; Dimbi *et al.,* 2003), its use in horticulture should also be promoted for control of African fruit flies. Although several isolates show promise for commercialization, as far as Kenyan agriculture is concerned the indigenous isolates such as *M. anisopliae* (ICIPE 20) may be the pathogen of choice because it is native and unlikely to raise public concern among regulatory agencies.

Sexually mature *B. inavdens* may lay over 1000 eggs and any control strategy that targets reduction in oviposition in field population should contribute to overall management of the insect. Although the effect of fungal infection on fruit fly fecundity and fertility is poorly documented, a number of recent laboratory studies have shown that infection by fungus may have adverse effects on fecundity and fertility. Work by Dimbi *et al*. (2003) demonstrated significant decrease in fecundity but not fertility on three fruit fly species, *C. capitata, C. cosyra and C. fasciventris* when infected with *M. anisopliae* Isolate ICIPE 62. Report by Quesada-Moraga *et al*. (2006) also demonstrate reduction in fecundity and hatchability at levels of 20.0 - 71.2% and 33.3 – 60.0% respectively in *C. capitata* adults treated with *M. anisopliae* isolate EAMa 01/58-su and *B. bassiana* EABb 01/103-Su. Castillo *et al*. (2000) demonstrated reduction in fecundity and fertility at levels of 40-50% and 50% respectively by *M. anisopliae*-treated females of *C. capitata* at $1x10^6$ conidia/fly compared to control. A similar significant difference in fecundity was exhibited when reproductive effect of *M. anisopliae* on female *Raghotelis indefferens* Curran was assessed (Yee and Lacey, 2005).

The precise mechanism surrounding the reduction in fecundity and fertility is unknown. However, entomopathogenic fungi affect host insects through a combination of events including mechanical damage by hyphal growth, nutrient depletion and production of toxins (Hajek and St Leger, 1994). Apart from effect of EPF on mortality by exhaustion of nutrients in the haemolymph and liberation of toxins, nutritional deficiency and toxic effect can affect development and behaviour of an insect especially in critical processes such as reproduction, which have high energy demands (Gulan and Granston, 2005). Colonization of tissues such as fat body (source of vitellogenins) and ovaries can result into disruption of follicular development (Hajek and St. Leger, 1994; Kaaya *et al*., 1991). Any of the above processes at a sub-lethal level may have negatively impacted on the reproductive system of the fruit flies, consequently reducing fecundity and fertility. Such secondary effect coupled with mortality may play a vital role in the management of insect pests. The ultimate aim of any fruit fly management is to reduce oviposition by gravid females and the overall effect of fungal infection on fecundity and fertility of *B. invadens* as reported in this study; and will be crucial in any suppression campaign for this pest.

Future practice of sustainable fruit fly suppression among subsistence farmers however will require affordable integrated management tools e.g. use of auto-inoculative devices that work as baits and laced with entomopathogen. This dissertation aimed at evaluating contamination devices made from different baiting materials and laced with dry conidia of *M. anisopliae* ICIPE 20; where flies will be infected with fungus as they land on open bait and escape to transmit disease to non-infected individuals of its population. The benefit of such IPM strategy would include reduced pesticide residues in food, safety for

humans; and increased biodiversity; although propagule survival under field conditions remain a challenge during field control due to ultra-violet B portion of solar radiation which is very detrimental (Inglis *et al*., 1995a). However, with fruit flies, uptake of conidia from auto inoculative devices placed under mango canopy is likely to maintain pathogen efficacy and become vital component of good control. Under these circumstances, inactivation of conidia would be slower because of protection of conidia from direct sunlight provided by plant canopy.

In the suppression study of *B. invadens* using 3 different bait stations (2% Nulure®, 10%DuduLure®) and DuduLure®) contaminated with conidia of *M. anisopliae,* results showed that flies did not land on any bait immediately when introduced into the cages where the contaminated baits were hang. Light direction was the first attractant followed by dispersive movements within the cage before landing on the baits. This behaviour has been reported as a common phenomenon among multivoltine species of fruit flies in which internal circadian rhythms control each activity and amount of time spent on it depending on age, sex and availability of mate (Fletcher, 1987; Aruna, unpblished data). Results of resident time spent on contaminated baits showed that over 80% of the flies landed on diluted fresh baits and diluted aged baits within a duration of 1 and 28 *seconds,* suggesting that there is considerable tolerance for fungus contaminated and aged bait station and the stations remained attractive to adult *B. invadens* when exposed in the field. Considerable microbial growth (mainly *Rhizopus* species) was observed on aging dry DuduLure bait station at 21 day after exposure in the in field but this did not deter attraction to the bait station. Similarly, results showed that dilution did not affect

attraction of old and fresh baits when exposed in the field and that bait modifications after registration of organic bait DuduLure® did not diminish bait function. These results are similar to those of Mangan (2002) in which dilution of a commercial bait GF-120, containing the bait spinosad did not affect attraction or toxicity to the flies for a period of three weeks. The short period that the flies spent on contaminated baits was found to be sufficient for them to pick lethal dose of propagules and cause mortality. Indeed as demonstrated earlier, flies that spent maximum of 30 seconds on the infection chamber picked an approximate dosage of 1×10^4 conidia, which induced 100% mortality by day 5. These results are in agreement with those of Furlong *et al.* (1995) who reported that diamondback moth *Plutella xylostella* (Lepidotera: Nuctidea) that spent one second in a fungus contaminated- pheromone bait caused lethal infection in the insect. The structural design of the 3 bait stations was similar and the fact that they were not sheltered enhanced attraction and infection efficiency on flies upon landing on baits.

Germination of conidia that were spread on 2% NuLure and 10% DuduLure baits remained high at 96% and 78% at 28 days of exposure in the field (Table 4.8). These bait stations were generally more superior to the dry DuduLure bait (6.1%). Hong *et al*. (2001) reported that for effective suppression of pests in the field, germination of 80% or more should be maintained. Survival of conidia in the environment unfortunately is influenced by several biotic and abiotic factors, amongst which ultra-violet-B component of solar radiation remains the most detrimental through damaging its DNA functions and drastically reducing viability (Inglis *et al*., 1996b; Carruthers and Soper, 1987; Ekesi *et al*., 1998, 2003; Fargues *et al*., 1996; 1997b; Zimmerman, 1982). In this bioassay, *M.*

anisopliae propagules on substrates were exposed in protected locations within the mango canopy, from which there was no direct solar radiation on propagules, hence prolonged persistence. However, Smits *et al*. (1996) reported that even within shaded areas, propagules showed decreasing conidial viability with time, as was the case with this experiment; meaning other array of abiotic and biotic factors could still affect the ability of these propagules to survive. Composition of substrate material of the bait station may have also been detrimental to survival of conidia but this was not assessed. During the microscopic observations for germination of ICIPE 20 on baits, *Rhizopus* was observed to grow on dry DuduLure bait station, colonized and out-competed the entomopathogen of interest. The 2-way anova supported this argument by demonstrating that although there was no significant effect of bait on viability of *M. anisopliae* conidia, baits can only be exposed in the field for a maximum of 4 weeks. Results of this study showed high germination levels in 2% NuLure and 10% DuduLure bait stations under fluctuating temperatures between 24.1 and 17.3°C (Table 4.3) under mango canopy. At Nguruman Dimbi *et al*. (2003) using *M. anisopliae* isolate ICIPE 20 reported viability of 70% after 4 weeks under fluctuating field temperatures between 23 °C and 35 °C under mango canopy. Percentage germination values obtained in this study were higher (78 - 96% after 28 days) than those reported by Dimbi *et al* (2003). Temperatures within the field where current study was carried out fluctuated between 17 - 24°C during the period of investigation compared to temperatures of 35°C under the mango canopy in Nguruman (Dimbi *et al.,* 2003). On the contrary, Maniania (2002) reported a drop in conidial viability of *M. anisopliae* isolate ICIPE 30 from 86% to 62% and 86% to 43% on contamination devices that were exposed in the sun and shade respectively between 0 and

31 days. Thus several biotic and abiotic factors may play an important role in determining survival of an entomopathogen in the environment.

Overall, high mortalities were exhibited from flies that were infected by fungus treated 2% Nulure- and 10% DuduLure baits. The performance of the 2 baiting stations were largely at par up to 14 days of exposure, but mortality in the 10% DuduLure bait (83.5%) was significantly higher than the 2% NuLure (64%) exposed for 21 days; and by 28 days 2% NuLure station caused significantly higher mortality of 89.8% against 47.6% in 10% DuduLure. Statistical analysis showed these values were still significantly higher than the fungus treated dry DuduLure bait station (Figure 4.4). The decline in pathogenicity over time cannot be correlated with viability data because high levels of viability were observed throughout the experimental period on both stations. The reasons for the poor pathogenicity yet high viability at 28 day is difficult to determine.

Currently, fruit fly suppression using baits rely on a weekly application of the food bait as spray to 1 meter square of the canopy. The results of this study demonstrate that liquid spray can be replaced with the new generation technique of application of bait stations either directly exposed or sheltered in protected devices. Because fungus contaminated old baits stations remained attractive to flies and high percentage germination of the fungal propagules on baiting stations were scored, repeated weekly application of the bait is not required thus minimizing both the cost of application and labor that is required in the deployment of the treatment.

5.1 Conclusion

After progressive screening experiment using several hyphomycete isolates, *Metarhizium anisopliae* isolate ICIPE 20 was selected as one of the most virulent isolates to *B. invadens.* Isolate ICIPE 20 can infect, kill, and cause secondary effects on reproductive potential (teratogenicity) of *B. invadens* by reducing egg fecundity, and percentage hatchability which play an important role on population dynamics of the pest. Evaluation of the candidate isolate *M. anisopliae* in baiting stations proved effective in causing mortality in adult *B. invadens*. The adaptability of bait station device will depend in part on field testing and its efficacy, the growers acceptance of the technology, extension service personnel and the public being aware of the existence of the alternative.

The facultative nature of *M. anisopliae* makes it easy to mass produce *in vitro* on cheap economical substrates and use it on target delivery baiting system. Since epizootics are relatively uncommon with *M. anisopliae,* the pathogen does not establish in populations of non-targets. Its potential use in auto dissemination device to infect fruit flies can substitute the problems associated with high loss of biodiversity usually experienced during inundative, classical and chemical control.

Recommendations

1. Apart from *M. anisopliae* isolate ICIPE 20, this study recommends use of 4 other isolates of *M. anisopliae* (ICIPE 21, 43. 62 and 69) and 2 isolates of *B. bassiana* (ICIPE 295 and 303) for future control studies on adult stages of *B. invadens* since they can cause infection and high rates of mortality to the flies. However, among the seven strains use of a native isolate *M. anisopliae* (ICIPE 20) in fruit fly suppression in the field is recommended because it is also an indigenous isolate and unlikely to raise public concern among regulatory agencies.

2. Bioassay results on effect of *M. anisopliae* (ICIPE 20) on fecundity and fertility indicated that the isolate significantly reduces egg fecundity and percentage hatchability. *Metarhizium anisopliae* (ICIPE 20) should be developed as a biological control agent against *B. invadens*.

3. Results on fruit fly suppression studies that used auto-inoculative baiting devices at semi-field trials (under mango canopy) indicate that 10% DuduLure and 2% NuLure® baiting stations that were treated with conidia of *M. anisopliae* retained significantly high percentage of germination and high virulence for a period of 4 weeks to adult flies which visited the baits. Future studies in fruit fly suppression should therefore use integrated pest management technology that will combine sanitation method and autoinoculative devices. Because of the prolonged persistence of such stations, repeated weekly application regime of the toxic bait is not required; instead, auto-inoculators will be serviced only once after every 4

weeks, minimizing both cost of application and labor that is required in the deployment of the treatment. Wooden sphere soaked in dilutions of commercial or locally developed bait and contaminated with spores of *M. anisopliae* provide a viable option for the management of *B. invadens* on mango.

4. Both bait dilutions (DuduLure® and Nulure®) remained attractive to *B. invadens* for over 4 weeks. Use of 10% DuduLure baiting stations is recommended to the subsistence farmers during fruit fly suppression because of its cheaper and locally available unlike NuLure®.

5. Since *B. invadens* and *C. cosyra* share same ecological niches in Kenya (Ekesi *et al.*, 2002; 2006), it may be possible to suppress both populations using *M. anisopliae* ICIPE 20 because the isolate has also demonstrated infection and high rates of mortality to native species of fruit flies.

REFERENCES

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

Agostino Bassi. 2000. The History of Italian parasitology, **98** (-34) pp. 3-131.

Ainsworth. 1973. Introduction and Keys to Higher taxa. *In*: "The Fungi". An advanced Treatise, (G.C. Ainsworth et al., Eds)., Academic press. Vol. **4B** pp 1 -7.

Allwood, A. J. 1996. Biology, and Ecology: Prerequisites for understanding and managing fruit flies (Diptera: Tephritidae). *In "*Management of Fruit Flies in the Pacific" (A.J. Allwood and R.A.I. Drew Eds.), pp 95-101. ACIAR Proceedings no. 76.

Allwood, A. J. 1997. Control strategies for fruit flies (Family Tephritidae) in the South Pacific. *In:* "Management of fruit flies in Pacific". A.J. Allwood and R.A.I. Drew (Eds). ACIAR Proceedings no. 76, pp 171-178

Alves, R. T., Bateman R. P. Chris P. and Simon R. L. 1998. Effects of simulated solar radiation on germination of *Metarhizium anisopliae* in different formulations. *Crop Protection* **17**(8)**,** 675-679.

Barnes, B. N., Eyles, D. K. and Franz G. 2004. South Africa's fruit fly SIT programme-the Hex River Valley pilot project and beyond. *In* "Proceedings of the 6th International Symposium on Fruit Flies of Economic importance. Stellenbosch, South Africa (N. Barnes *Eds.*), pp131-141. ARC.LNR

Bateman M.A.1972. The ecology of Fruit flies. *Annual Review Entomology* **17**, 493-518.

Bidochka M. J. Walsh, S. R.A., Ramos, M. E., St Leger R. J., Silver J. C. and Roberts D. W. 1996. Fate of biological control introductions : Monitoring and Australian fungal pathogen of grasshoppers in North America. Proc. Nall Acad. Sci. USA **93**: 918-921.

Butt, T. M., Carreck N. L. Ibrahim L. and Williams I. H. 1998. Honey bee mediated infection of pollen beetle (*Meligethes spp*) by insect-pathogenic fungus, *Metarhizium anisop*liae. *Biocontrol Science and Technology:* **8,** 533-538.

Butt T.M., Jackson C. and Magan, N. 2001. Introduction- Fungal Biological Control Agents: Progress, Problems and Potential. *In*: " Fungi as Biocontrol Agents " T. M. Butt, C. Jackson and N. Magan (Eds), pp 1-8.

Castillo, M. A., Moya, P., Hernandez, and Yufera E. P. 2000. Susceptibility of *Ceratitis capitata* Wiedemann (Diptera:Tephritidae) to entomopathogenic Fungi and their Extracts. *Biocontrol* : **9**, 274-282.

Carruthers R. I., Larkin, T. S. and Firstencel H. 1992. Influence of thermal ecology on the mycosis of a rangeland grasshopper. *Ecological Society of America:* **73**(1), 190-204.

Carruthers R. I., and Soper, R. S. 1997. Fungal diseases. In: J. R. Fuxa and Y. Tanada (Eds). Epizootiology of insect diseases. John Wiley and Sons (Eds). pp 357-416New York, USA,.

Cherry, A. J.,Jenkins N. E., Heviefo G, Bateman R. and Lomer C. J. 1999. Operational and economic analysis of West Africa pilot scale production plant for aerial conidia of *Metarhizium* spp. for use as Mycoinsecticide against Locusts and Grasshoppers. *Biocontrol Science and Technoly* **9**, 35-51.

Clarke, A. R., Karen, F. A., Amy, E. C., John, R. M., Raghu S., George, K. R. and David, K. Y. 2005. Invasive phytophagous pest arising through a recent tropic evolutionary radiation: *Bactrocera dorsalis* complex of fruit flies. *Annual Review of Entomology:* **50,** 293-319.

Copeland, R. S., Wharton, R. A., Luke, Q., Meyer M., Lux S., Zenz, N., Machera P. and Okumu M. 2006. *Annals of Entomological Society of America,* **99** (2) 261-278.

Damfa, A. and Van Der Valk, G. H. 1999. Laboratory testing of *Metarhizium* spp and *Beauveria bassiana* on Sahelian non-target arthropods. *Biocontrol Science and Technology*. **9**, 187-198.

Dimbi, S., Maniania, N. K., Lux, A, Ekesi, S. and Mweke, J. K. 2003. Pathogenicity of *Metarhizium anisopliae* (Metsch) Sorkin and *Beauveria bassiana* (Balsano) vuillamin to the three adult fruit fly species; *Ceratitis capitata* (Weidmann), *C. rosa* var fasciventris (Karsh) and *C. cosyra* (Walker) (Diptera: Tephritidae). *Mycopathologia* **156,** 375-382.

Dimbi, S., Maniania, N. K., Lux, S. and Mweke, J. K. 2004. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African fruit flies. *BioControl* **49**: 83-94, 2004.

Drew, R.A.I., Tsuruta K. and White I. M. 2005. A new species of pest (Diptera: Tephritidae:Dacinae) from Sri Lanka and Africa. *African Entomology*. **13** (1), 149-154.

Ekesi, S., Maniania, N. K. and Ampong, N. and Onu. 1998. Potential of the entomopathogenic fungus *Metarhizium anisopliae* (Metsch) Sorokin. For control

of legume flower thrips, *Megalurothrips sjostedti* (Trybom) on cowpea in Kenya. *Crop Protection* **17** (8) 661-668.

Ekesi S., Maniania N. K., and Lwande. 2000. Susceptibility of *Megalurothrips sjostedti* developmental stages to *Metarhizium anisopliae* and effects of infection on feeding, adult fecundity egg fertility and longevity. *Experimentalis et Applicata* **94**, 229-236.

Ekesi, S., Maniania, N. K. and Lux, S. A. 2002a. Mortality in three African fruit fly puparia and adults, caused by Entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. *Biocontrol Science and Technology*. **12**, 7-17.

Ekesi, S. and Maniania, N. K. 2002b. *Metarhizium anisopliae*: an effective biological control agent for the management of thrips in horti- and floriculture in Africa. In: *Advanced in Microbial control of insect pests.* pp 65- 180.

Ekesi, S., Maniania, N. K. and Lux, S. 2003. Effect of soil temperature and moisture on survival and fertility of *M. anisopliae* to four tephritid fruit fly puparia. *Journal of Invertebrate Pathology*. **83,** 157-167.

Ekesi, S., Maniania, N. K. and Mohamed S.A. Lux, S. 2005. Effect o soil application on different formulations of *Metarhizium anisopliae* on African fruit flies and their associated endoparasitoids. *Biological control* **35**, 83 -91.

Ekesi, S., Nderitu, P. W. and Rwomushana H. C. 2006. Field infestation, Life history and Demographic parameters of fruit fly *Bactrocera invadens* (Diptera: Tephritidae) in Africa. *Bulletin of Entomological Research* **96**, 379 – 386.

Fargues, J., Goettel M. S., Smits N., Ouedraogo, A., Vidal, C. Lacey, L.A. and Rougier, M. 1996. Variability in susceptibility in susceptibility to simulated sunlight of condia among isolates of entomopathogenic Hyphomycetes. *Mycopathologia.* **135**: 171-181.

Fargues, J. M., Goettel M. S., Ouedraogo, A., Goettel, M. S. and Lomer C. J. 1997b. Effects of temperature on relative humidity and inoculation method on susceptibility of *Schistocerca greagaria* to *Metarhizium flavoviridae*. *BioControl Science and Technology*. **7**: 345-356.

FAO Corporate Document Repository, 1998.

FAO IG –SG TF. 1999. Agricultural trade and food security: Agricultural factsheet. Third ministrerial conference. Rome FAO.

Feng, M. G. and Johnson, J. B. 1990. Relative susceptibility of six isolates of *Beauveria bassiana* on *Diuraphis noxia* (Homoptera: Aphididae). *Environmental Entomology* **19**, 785-790.

Ferron, P. 1978. Biological Control of Insect pests by Entomogenous Fungi. *Annual Review of Entomology*, **33**: 409-443.

Ferron, P. 1981. Pest control by Fungi *Beauveria bassiana* and *Metarhizium anisopliae*. *In*: "Microbial control of Pests and Plant Diseases 1970-1980, Academic Press London (H.D. Burges Eds) pp 465-476.

Ferron, P., Aeschlimann, P., Marchal, M. and Soares G., 1985. Occurrence of pathogenicity of *Beauveria bassiana* infesting larval *Sitona discoideus* (Coleoptera: *Curculionidae Entomophaga* **30**: 73-82.

Fletcher, B. S. 1987. The biology of Dacine fruit flies. *Annual Review of Entomolog*, **32**: 115-144.

French, C. 2005. The new invasive *Bactrocera* species; *In* "Insect control Newsletter" No. **(65)**". pp 19-20. International Atomic Agency, Vienna, Austria.

Furlong, M. J., Pell, J. K., , P. C., and Seyd, A. R. 1995. Field and laboratory evaluation of Ong sex pheromone trap for autodissemination of the fungal entomopathogen *Zoophthora radicans* (Entomophthorales) by te diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). *Bulletin of Entomological Research*, **85**, 331-337.

Garcia, A. S. Messias, C. L., Souza, de and H. M. L. Piedrabuena, A. E. 1984. Patogenicidade de *Metarhizium anisopliae* a *Ceratitis capitata* (Wiedeman) (Diptera : Tephritidae). *Revista Brasileira de Entomologia* **28** : 421- 428.

Goettel, M. S. and Johnson, D. L. 1992. Environmental impact and safety of fungal biocontrol agents. In "Biological control of Locusts and Grasshoppers*"* (C. J. Lomer and C. Prior Eds), pp. 356-361. *CAB International, Wallingford.* UK.

Goettel, M. S. and Jaronski, S. T. 1997. Safety and Registration of Microbial agents for control of Grasshoppers and Locusts. In "Microbial control of Grasshoppers and Locusts". *Memoirs of Entomological Society of Canada,* M. S. Goettel and D. L. Johnson (Eds). Volume **171:** pp 82-99.

Goettel M. S., Inglis G. D and Wraight S. P. 2000. Fungi. *In*: "Field Manual techniques in Invertebrate Pathology. Application and Evaluation of pathogens for control insects and other Invertebrate prests. L. A.Lacey and H, K, Kaya (Eds) pp. 255-282. Kluwer Academic. Dordrecht.

Gomez, K. A. and Gomez, A.A. 1984. Statistical Procedures for Agricultural Research. Wiley, New York, 680 pp. Wiley, New York.

Gulan P. J., Granston P. S. 2005. The insects: An outline of entomology (3rd ed). Oxford Blackwell Publishing, pp 61-65.

Hajek, A. E. and St. Leger R. J. 1994. Interactions between fungal pathogens and insect hosts. *Annual Review of Entomology*, **39**, 293-322.

Hajek, A. E., Buttler, L. and Wheeler. 1995. Laboratory bioassays testing the host range of the gypsy moth fungal pathogen *Entomophaga maimaiga*. *Biological Control* **5**: 530- 544.

Hajek, A. and Goettel, M.S. 2000. Guidelines for evaluating effects of entomopathogens on non-target organisms. "*In* Field Manual Techniques in Insect Pathology" (L.A. Lacey and H.K. Kaya Eds), pp. 101-113. Kluwer Academic Publishers, London.

Hall and Papierok. Fungi as biocontrol agents of arthropodsn of agricultural and medical importance. *Parasitology*, 1982: **84**: 205 -240.

HCDA, 2002. Classification of cultivars according to maturity and seasons *In*: Mango growing in Kenya. M. A. Nyamu and T. Simons (Eds.). pp 23-86.

HCDA. 2004. Horticulture Industry in Kenya. Waithaka M. and Noah, (Eds). pp 7-9.

HCDA. 2007. Progress report on Good Agricultural practices, MOA/HCDA/JICA-training team Horticultural crops Development Authority News.

Hong, T. D. Gunn J. Ellis R. H., Jenkins N. E. and Moore D. 2001. The effect of storage environment on the longevity of conidia of *Metarhizium flavoviridae* Gams and Rozsypal and interactions with temperature. *BioControl Science and Technology* **6**, 51-61.

Humber, A.H. Fungi Identification. 1997. *In "*Manual of techniques of Insect Pathology" L. Lacey, (Ed.), pp 153-185. Academic Press, New York.

Humber R. A. 1992. Collection of entomopathogenic fungal cultures: Catalogue of strains, USDA. ARS/ ARS-110, 117 pp.

Inglis, G. D. Goettel, M. S. and Johnson, D. L. 1993. Persistence of entomopathogenic fungus *Beauveria bassiana* on phylloplanes of crested wheat grass and alfafa. *Biological Control* **3**, 258-270.

Inglis, G. D. Goettel, M. S. and Johnson, D. L. 1995a. Influence of ultra violet light protectants on persistence of entomopathogenic fungus, *Beauveria bassiana*. *Biological Control.* **5**, 581-590.

Inglis, G. D., Goettel, M. S. and Johnson, D. L. 1996a. Effects of temperature on thermoregulation on mycosis by *B. bassiana* in grasshoppers. *Biological Control,* **7**, 131-139.

Inglis, G. D., Johnson, D. L. and Goettel, M. S. 1996b. Effect of bait substrate and formulation on infection of grasshopper nymphs by *Beauveria bassiana*. *BioControl Science and Technology*. **6**, 35-50.

Inglis, G. D., Johnson, D. L. and Goettel, M. S. 1997b. Field evaluation of *Beauveria bassiana* against grasshoppers: Influence of temperature and light exposure on mycosis. *Environmental Entomology,* **26**: 400-409.

Inglis, G. D., Goettel M. S. Butt T. M. Strasser, H. 2001. Use of Hyphomycetous fungi for managing insect pests. *In*: Fungi as Biocontrol Agents (T.M. Butt, C. Jackson and N. Magan Eds.) CAB *International*. Pp 23-69

Jaronski, S. T., and Goettel, M. S. 1997. Development of *Beauveria bassiana* for control of grasshoppers and locusts. *In: "*Microbial control of Grasshoppers and locusts". Goettel M.S. and D.L. Johnson (Eds) and Memoirs of Entomoligical Society of Canada, **171,** pp. 225-237.

Jenkins, N. E. and Prior, C. 1993. Growth and formation of true conidia by *Metarhizium flavoviridae* in a simple liquid medium. *Mycological Research*, **97**, 1489 -1494.

Jenkins, N. E., Gabriel, H., Langewald, J., Cherry A. J., and Lomer C. J. 1998. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information* **19** (1) 21N-31N.

Kaaya, G. P. Kokwaro E. D. 1991. Mortalities and lession caused by *Beauveria bassiana* and *Metarhizium anisopliaie* in adult tsetse, *Glossina morsitans*. *Discovery and innovation,* **3**, 55- 60.

Kaaya, G. P., and Munyinyi, D. M. 1995. Biocontrol potential of entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae* for tsetse flies (*Glossina spp*.) at development sites. *Journal of Invertebrate Pathology*., **66,** 237-241. .

Kerwin J. L. and Peterson E. E. 1997. Fungi: Ooomycetes and Chytridiomycetes. *In*: Manual of Techniques in Insect Pathology. L. Lacey (Eds.) pp 251-268.

Klein, M. G. and Lacey L. A. 1999. An attractant trap for auto-dissemination of entomopathogenic fungi into populations of Japanese beetle *Popilla japonica* (Coleopterta: Scarabaidae). *BioControl Science and Technology* **. 9,** 151-158.

Konstantepoulo, M. A. and Mazomers, B. E. 2005. Evaluation of *Beauveria bassiana* and *B. Brongniartii* strains and four wild type fungal species against adults of *Bactrocera olae* and *C. capitata*. *Biological Control,* 32: 293-305.

Lacey L., Undeen, A.H. 1986. Microbial control of black flies and mosquitoes. *Annual Review of Entomoolgy,* **31**: 265-296.

Lacey, L. A. and Goettel, M. S. 1995. Current developments in microbial control of insect pests and prospects for the early 21st century. *Entomophaga*, **40**, 3-28.

Lomer C. J. and Prior, C, and Kooyman. 1997. Development of *Metarhizium* species for the control of grasshoppers and locusts (M. S. Goettel and D. L. Johnson Eds) pp 265-286. *Memoirs of Entomological Society of Canada,* Vol. **171,** 265-286.

Lux, S. A., Copeland, R. S. White, I. M., Manrakhan, A and Billah, M. K. 2003. "A new invasive fruitfly species from *Bactrocera dorsalis* (Hendel) group detected in East Africa. *Insect Science and its Application,* **23** (4), pp. 355-361.

Mangan R. L. and Moreno D. S. 2002. Dilution and persistence of baits and safer pesticides for spray application. *In:* Proceedings of 6th international fruit-fly symposium, 6th May 2002, Stellenbosch, South Africa. pp. 305-312.

Maniania N. K. 2002. A low cost contamination device for infecting adult tse-tse flies, *Glossina* species with the entomopathogenic *Metarhizium anisopliae* in the field. BioControl Science and Technology, **12**, 59-66.

Milner R. J. 1982. On occurrence of pea aphids, *Acyrthosiphoin pisum*, resistant to isolates of *Erynia neoaphidis*. *Entomologia et Experimentalis et Applicata,* **32**, 23-27.

Milner R. J. 1985. Distribution in time and space of resistance to the pathogenic fungus *Erynia neopaphidis,* in the pea aphid *Acyrthosiphoin pisum*. *Entomologia Experimentalis et Applicatta*, **37**, 232-240.

Milner, R. J., Staples J. A., Hartley, T. R., Lutton, G. G. 1998. The selection of an isolate of the hyphomycyte fungi *Metarhizium anisopliae* for control of termites in Australia. *Biological control*, **11**: 240-247.

Ministry of Agriculture. 2002. Horticulture annual report 2001. Nairobi , Kenya

Mochi D. A. Monteiro A. C., Sergio A., Bortoli D., Doria H.O.S.Barbosa J. C. 2006. Pathogencity of *Metarhizium anisopliae* for *Ceratitis capitata* (Wied.) (Diptera: Tephritidae) in soil with different pesticides. *Neotropical Entomology* **35**(3) 382-389.

Moore, D. and Prior. 1993. The potential of Mycoinsecticides. *BioControl News and Information*, **14**: 31N-40N.

Mohamed, A. K. A., Sikorowski, P. P. and Bell V. J. 1977. Suceptibility of *Heliothis zea* larvae on *Nomuraea rileyi* at various temperatures. *Journal of Invertebrate Pathology*, **30**, 414-417.

Mullens B. A. Rodziguez J. L and Meyer J. A. 1987. An epizootiological study of *Entomophthora musca*e in mucosoid fly populations on southern California poultry facilities on *Musca domestica. Hilgardia* **55** (3), 1-41

Mullens B. A. 1989. Cross transmission of Entomophthora muscae (Zygomycete: Entomophthoracae) among naturally infected muscoid fly (Diptera: Muscidae) hosts. *Journal of Invertebrate Pathology*, **53**: 272-275.

Papierok B. and Hajek A. E. 1997. Entomophthorale. In: Manual of techniques in Invertebrate Pathology." Lacey L. A. (Eds). Academic Press, London. pp. 187-212.

Pell, J. K., Wilding, N., Player, A. L. and Clark, S. J. 1993a. Selection of an isolate of *Zoophthora radicans* Brefeld (Zygomycetes: Entomopthorales) for biocontrol of diamond back moth, *Plutella xylostella* (Lepidotera: Yponomentidae). *Journal of InvertbratePathology*, **61,** 75-80.

Pell, J. K., Macanley, E. D. M. and Wilding, N. 1993b. A pheromone trap for dispersal of the pathogen *Zoophthora radicans* (Zygomycetes: Entomophthorale) amongs population of the diamond back moth, *Plutella xylostella* (Lepidotera: Yponomentidae). *BioControl Science and Technology*, **3**, 315-320.

Pena, J. E., Monyuddin, A. I. and Wysoki, M. 1998. A review of Pest management Situation in Mango Agrosystems. *Phytoparasitica* **26** (2), 1-20.

Poprawski, T.J., Marchal, M. & Roberts, P.H., 1985. Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early instar stages to five entomopathogenic hyphomycetes. *Environmental Entomology* **14**, 247-253.

Prokopy, R. J., Papaj, D. R., Hendrichs, J. & Wong, T. T. Y. 1992. Behavioural responses of *Ceratitis capitata* flies to bait spray droplets and natural food. *Entomologia Experimentalis et Applicata* **64**, 247-257.

Prokopy, R. S., Miller, N. W,, Pienero, J. C., Janes, D. B., Lind, C. T., Leslie O. and Roger I. V. 2003. Effectiveness of GF-120 fruit fly bait spray applied to border area plants for control of Melon flies (Diptera: Tephritidae*). Journal of Economic Entomology*, **96** (5); 1485-1493.

Quesada-Moraga E. Ruiz-Garcia and Santiago-Alvarezb beta, C. 2006. Laboratory evaluations of entomopathogenic fungi *Beauveria bassiana* and *Metarhiziuum anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Economic Entomology,* **99**(6), 1955 - 1966.

Roberts, D. W. and Humber, R. A. 1981. Entomogenoeous fungi. *In:* Biology of conidia; fungi Vol**. 2** pp. 201-236. G. T. Cole and W. B. Kendrick (Eds). Academic Press New York.

Rwamushana, I; Ekesi S., Ogol C. K. P. O. and Gordon I. 2008. Effect of temperature on development and survival of mature *Bactrocera invadens* (Diptera: Tephritidae) *Journal of Applied Entomology*. **132**: 832-839.

Rwamushana, I., Ekesi S. Gordon I., Ogol C. K. P. O. 2008. Host plants and host plant reference studies for *Bactrocera invadens* (Diptera: Tephritidae) in Kenya, A new invasive fruit fly species in Africa. *Annual Entomological Society of America,* **10** (2): 331-340.

Samira A. M., Sunday E., and Richard H. 2008. Laboratory evaluation of parasitism of *Bacrtocera invadens* and five *Ceratitis* species by opine parasitoid *Fopius arisanus* and *Diachasmimorpha longicudata*. In: *Current trends in Biological Control. 1st meeting of team:* Palma of Mallorca. 7-8 April, 2008.

SAS Institute Copyright © (1999-2001). SAS/STAT User guide Release 8.2 .Inc. Cary, NC, USA.

Samuels R.I. Reynolds S.E. and Charnley 1988. Calcium channel actovation of insec muscle by dexrusins insecticidal compounds produced by the entomogenous fungi *Metarhizium anisopliae*. *Comparative Bochemistry and Physiology* **90C**, 403- 412.

Siegel, P. J. 1997. Testing pathogenicity, infectivity of entomopathogens to mammals. IN manual of techniques in Insect Pathology (Lawrence L. (Ed). pp 325-336. Academic Press, New York.

Smits, N., Fargues, J., Rougier, M.Giujet, R. and Itier, B. 1996. Effects of temperature and solar radiation interactions on the survival of quiescent conidia of entomopathogenic hyphomycete Paecillomces fumosoroceus (Wize) Brown and Smith. *Mycopathlogia* **135**: 163-170.

St Leger, R. J., Cooper, R. M. and Charnely A. K. 1986a. Cuticle degrading enzymes of entomopathogenic fungi: Cuticle degradation *in vitro* by enzymes from entomopathogens. *Journal of Invertebrate Pathology*, **47**: 167-177.

St Leger, R. J., Butt, T. M., Goettel M. S. Staples R. C., and Roberts D. W. 1989. Production *in vivo* of appressoria by the entomopahogenic fungi *Metarhizium anisopliae* . *Experimental mycology,* **13**: 274-288.

St Leger, R. J., Staples, R. C. and Roberts, D. W. 1992a. Molecular cloning regulatory analysis of cellular degrading protease structural genes from the entomopathogenic fungal *Metarhizium anisopliae*. *European Journal of Biochemistry* **204:** 991-1001.

St Leger, R. J, Staples R. C. and Roberts D. W. 1992a. Entomopathogenic isolates of *Metarhizium anisopliae*, *Beauveria bassiana* and *Aspergillus flavus* produce multiple extracellular chitinase isozymes. *Journal of Invertebrate Pathology***, 61**: 81-84.

St Leger, R, J. 1993. Biology and mechanisms of insect cuticle invasion by Deuteromycete fungal pathogens.pp 211-229. *In*: Parasites and Pathogens of insects Vo.l **2**, : London Academic Press.

Steiner, L. F., Mitchell W.C., Harris, E. J., Kozuma T. T. and Fujimoto, M. S. 1965. Oriental fruit fly eradication by male annihilation. *Journal of Entomology,* **58**: 961-964.

Steinhaus E. A. 1975. Infection and epizootiology. *In" Principles of Insect Pathology"* (E. A. Steinhaus Ed*), pp 166-189* McGraw-Hill Ed. Book co. Inc. New York.

Vargas, R. I., Miller, N. W. and R. J. Prokopy. 2002. Attraction and feeding responses of Mediterranean fruit fly and a natural enemy to protein baits laced with two novel toxins, Phloxine B and Spinosad . *Entomologia Experimentalis et Applicata* **102**, 273-282.

Vega, F. F., Doud, P. F. Lacey, L. A., Pell, J. K. Jackson D. M. and Klein M. G. 2000. Dissemination of Beneficial microbial agensts by insetcs. In: "Field Manual techniques in Invertebrate Pathology. Application and Evaluation of pathogens for control insects and other Invertebrate prests. L. A. Lacey and H, K, Kaya (Eds) pp. 152-177. Kluwer Academic. Dordrecht.

Vestaergaard, S., Cherry A., Keller, S. and Goettel, M. 2003. Safety of Hyphomycete fungi as microbial control agents. *In:* "Environmrntal Impacts of Microbial Insecticides, pp. 35-62. (H. M. T. Hokkanen and A.E. Eds.) Hajek Kluwer Academic publishers", Netherlands.

Wharton R. A., Trostle, M. K., Messing, R. H., Copeland, R. S., Kimani-Njogu, S. W., Overholt, W. A., Mohamed, S. and Sivinski, J. 2000. Parasitoids of medfly, *Ceratitis capitata* and related tephritids in Kenyan coffee: a predominantly koinombiont assemblage. *Bulletin of Entomological* research **90**, 517-526.

White, I. M. and Elson-Harris, M. M. 1994. Fruit flies of Economic importance. Their identification and bionomics. Wallingford, UK: CAB International 610pp.

Yee, W. L. and Lacey, L. A. 2005. Mortality of different stages of *Rhagotelis indifference* (Diptera: Tephritidae) exposed to the entomopathogenic fungus *Metarhizium anisopliae*. *Journal of entomology*. Soc. **40**: 167-177.

Zimmerman, G. 1982. Untersuchungen zur Wirkung von *Metarhizium anisopliae* (Metsch.) Sorok. Auf Eier und schlupfende Eilarven von *Otiorhynchus sulcatus* F. (Col., Curculionidae). *Z. Angew*. *Entomology*. **93**, 476-482.