Development of a fungal based-product for the control of Western flower thrips *Frankliniella occidentalis* (Pergande)

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DECLARATION BY CANDIDATE

This thesis is my original work and has not been presented in any other University

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DECLARATION BY SUPERVISORS

We attest the work reported in this thesis was accomplished by the candidate under our supervision. We have read and approved this thesis for submission.

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DEDICATION

I dedicate this thesis to my late Daddy Dr. Abdoulaye Niassy (1953-2006) a Senegalese entomologist, former ARPPIS.

Make me say some few words to pay tribute to your loving memory. I cannot find ways to express my gratitude for bringing me up in the realm of science particularly in entomology from my most tender childhood and for showing me the secret of hard work perseverance and generosity. To me this thesis is a “dream come true”; endeavouring a Ph.D degree this field of entomology at the finest level and be among the select-few Africans elite who achieved their research degree at *icipe* in Kenya.

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ABSTRACT

The Western Flower Thrips (WFT) *Frankliniella occidentalis* (Pergande) is a major constraint to many cultivated crops in the world, causing important economical yield losses. They cause direct damage on plants and contribute exclusively in the propagation of tospovirus to plant.

Thrips control is essentially based on the use of synthetic chemical pesticides; however most thrips species including *F. occidentalis* are resistant to a wide group of chemical pesticides. Hence there is the need to develop alternative strategies that are environmentally friendly. Entomopathogenic fungi are among the options being considered as a promising alternative for *F. occidentalis* management. The aim of this study was to develop a fungal based-product for the control of *F. occidentalis*.

Larval stages of *F. occidentalis* are often considered to be refractory to fungal infection as compared to the adult stage; hence, screening of 10 isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin and 8 of *Beauveria bassiana* (Balsamo) Vuillemin was carried out in order to select virulent entomopathogenic fungal isolate(s) for their control. The most virulent isolates were compared in terms of conidial production and genetic polymorphism. All the fungal isolates tested were pathogenic to the second-instar larvae of WFT, causing mortalities ranging between 24 and 56%. *M. anisopliae* isolates ICIPE 7, ICIPE 20, ICIPE 69 and ICIPE 665 had the shortest LT_{50} values, causing mortalities within 8.0-8.9 days. *M. anisopliae* isolates ICIPE 69, ICIPE 7 and ICIPE 20 had the lowest LC_{50} values of 1.1×10^7, 2.0×10^7 and 3.0×10^7 conidia ml^{-1}, respectively. In terms of conidia production, *M. anisopliae* isolate ICIPE 69 produced significantly more conidia than the other two isolates. ITS sequence comparison indicated that ICIPE 69 differed from the other isolates for 2bp corresponding to a restriction site SfoI which could be due to the difference in geographical origin. These results coupled with previous studies on its virulence and field efficacy to other thrips species

make *M. anisopliae* isolate ICIPE 69 a suitable candidate for development as fungal biopesticide for thrips management.

In order to understand the underlying genetic mechanism behind virulence of the *M. anisopliae* ICIPE 69, 8 isolates of *M. anisopliae* (ICIPE 7, ICIPE 20, ICIPE 30, ICIPE 41, ICIPE 62, ICIPE 63, ICIPE 69 and ICIPE 78), which had previously shown to be pathogenic to *F. occidentalis,* were characterized using chitinase genes (*chi1*, *chi2*, *BbTrch* and *chi4*). Results suggest that although chitin digestion is critical in fungal infection, the use of chitinase genes for genotyping might not be appropriate for virulence characterisation.

The compatibility of *M. anisopliae* isolate ICIPE 69, with 12 agrochemicals in an integrated pest management perspective was assessed, under laboratory conditions. The agrochemicals included 5 insecticides, one botanical insecticide, 2 acaricides, and 3 fungicides, commonly used in French bean production in terms of conidia production, vegetative growth and mycelial mass. The insecticides abamectin and imidacloprid were highly compatible with *M. anisopliae*; thiamethoxam was compatible whereas azadirachtin and L-cyhalothrin were toxic to the fungus. The acaricide spiromesifen was moderately toxic while the fungicides carbendazim, probineb and copper-hydroxide were very toxic to the fungus. The association of the *M. anisopliae* ICIPE 69 with imidacloprid or thiamethoxam did not result in any synergistic or antagonistic effects on larvae of *F. occidentalis.* However, the combination of lower concentrations of both fungus and thiamethoxam resulted in shorter LT_{50} values compared to individual treatments. Our results suggest that application of the fungus along with agrochemicals like imidacloprid and thiamethoxam can be an efficient IPM strategy in pest control.

The intra and inter specific effects of host-plant on the virulence of *M. anisopliae* on *F. occidentalis* was evaluated in the laboratory. Three varieties of French bean, *Phaseolus vulgaris* L. var. *Alexandria*, *Julia* and *Samantha* and Snow pea *Pisum sativum* L. were

selected for the study. *F. occidentalis* cohorts (Second-instar larvae cohorts of WFT were reared for three generations before the bioassays. They were then exposed to pods of host plants previously sprayed with three concentrations of *M. anisopliae* (1×10^6; 1×10^7; 1×10^8 conidia ml^{-1}). There were no significant differences in mortality between host-plants. However, the comparison of LT_{50} values showed that thrips were more susceptible when reared on *Alexandria*. Inter specific comparison showed that there were no significant differences in LT_{50} between snow pea and the French bean variety *Julia*. Results suggest that intra-specific and inter-specific host-plant effects need to be taken into consideration during pest management programmes.

The performance of an autoinoculation device for *M. anisopliae* was evaluated in field cage experiments for the control of *F. occidentalis* in French bean. Treatments consisted of an autoinoculation device with semiochemical (LUREM-TR a commercial thrips attractant), a *M. anisopliae* treated device without semiochemical and a fungus-free device as control. Parameters assessed included conidial acquisition by thrips, thrips mortality, and conidial persistence in the device. The overall mean of conidia acquired by single insect was higher ($5.0 \pm 0.6 \times 10^4$ conidia/insect) in the fungus-treated semiochemical-baited device than in the device without semiochemical ($2.2 \pm 0.4 \times 10^4$ conidia/insect). The overall thrips mortality was also higher in the fungus-treated semiochemical-baited device (59.3 ± 3.9%) as compared to the device without the semiochemical (41.7 ± 3.5%). Viability of *M. anisopliae* conidial was not affected in autoinoculation device without semiochemical until 7 days after treatment. However, it was considerably affected in the autoinoculation device with semiochemical, where the conidial viability decreased from 81.0 ± 1.3%, 2 days post-inoculation to 6.5 ± 1.1%, 7 days post-inoculation. There was a positive correlation between mortality and conidial persistence and a negative correlation between conidial persistence and conidial acquisition, suggesting that the semiochemical volatiles significantly affected the

conidial viability which in turn resulted in significant reduction in thrips mortality despite the highest conidial acquisition in the fungus-treated semiochemical-baited device. Thrips density per plant was significantly reduced in both the autoinoculation device with semiochemical (autoinoculation adults/plant) autoinoculation device without semiochemical (8.7 ± 1.7 adults/plant) and autoinoculation (6.6 ± 1.4 adults/plant) as compared to the control (19.8 ± 2.6 adults/plant). These results demonstrate the prospects of autoinoculation device strategy for dissemination of *M. anisopliae* in the control of thrips, particularly in screenhouses.

The effects of the entomopathogenic fungus *M. anisopliae* (ICIPE 69) infection on *T. tabaci* feeding and its competence to vector IYSV in onion plant were investigated. Newly-emerged *T. tabaci* larvae were allowed to feed on IYSV-infected onion leaves in order to get infection and to become adults. Newly-emerged adult *T. tabaci* were treated with three sublethal concentrations of *M. anisopliae* (10^5; 10^6 and 10^7 conidia ml^{-1}) and placed in a sterile 9-cm Petri dish on a clean onion leaf-disc and allowed to feed for 5 days. The numbers of feeding punctures were recorded daily for five days. The viral titer in adult insects and onion leaves was measured using DAS ELISA technique. Infection by *M. anisopliae* significantly reduced (ANOVA: $F_{3, 480} = 50.4$; $P < 0.0001$) the feeding punctures by adult *T. tabaci.* Although there was no significant effect of *M. anisopliae* infection on IYSV titer in adults *T. tabaci*, virus transmission was significantly reduced (ANOVA: $F_{3,240} = 14.4$, $P = 0.0001$) in *M. anisopliae*-infected insects compared to the control. There was a significant positive correlation between feeding punctures and IYSV transmission in onion leaf-disc, suggesting that *M. anisopliae* application in addition to the effective control of the vector, *Thrips tabaci* can also influence IYSV propagation by reducing *T. tabaci* feeding punctures. However, further investigations are needed to determine effect of *M. anisopliae* on IYSV acquisition by *T. tabaci*.

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ANOVA Analysis of Variance

BCA Biological Control Agent

BLAST Basic Local Analysis Search Tool

DAS-ELISA Double Antibody Sandwich ELISA

Df Degree of Freedom

EPN Entomopathogenic Nematodes

ICIPE International Centre of Insect Physiology and Ecology

IPM Integrated Pest Management

IYSV Iris Yellow Spot Wilt Virus

JKUAT Jomo Kenyatta University of Agriculture and Technology

L: D Light-Darkness photoperiod proportion

RH Relative Humidity

SAS Statistical Analysis System

SDA Sabouraud Dextrose Agar

SE Standard Error

SNK Student-Newman-Keuls

WFT Western Flower Thrips

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* Pergande, Thysanoptera: Thripidae) is among the most important economical thrips pest of cultivated crops including cut flowers, vegetables and fruits throughout the world . Besides direct damages by feeding and oviposition in the plants, WFT is an efficient vector of tospoviruses diseases to plants (Baker *et al.*, 1993). The spread of the WFT is related to the increased global trade across international boundaries. The wind also seems to play an important role in the distribution of this pest (Kirk and Terry, 2003).

Over the past decades, synthetic chemical pesticides which are toxic to humans and hazardous to the environment have been the major control method of managing thrips pests (Lewis, 1997a; Nderitu *et al,* 2008; Waiganjo *et al.*, 2006). In addition *F. occidentalis* has developed resistance to many classes of synthetic chemical pesticides (Lewis, 1997a; Jensen, 2004). Requirements of strict compliance to maximum residue limit (MLR) norms by importing countries in the developed world limits the use of pesticides in thrips management.

Other techniques such as plant sanitation, the use of varietal resistance and intercropping are also used to limit thrips population to below economical thresholds levels (Waiganjo *et al.*, 2006). The use of colored sticky traps and semiochemicals is also being developed for thrips monitoring, mass-trapping and population control (Teulon and Penman 1992; Teulon *et al.*, 2010; Berry *et al.*, 2010).

Although *F. occidentalis* has many natural enemies including predatory mites, parasitoids, entomopathogens (Navon and Aschers, 2000; Loomans and Murrai, 1997; Blaeser *et al.*,

2002; Venzon *et al.*, 2002), the use of natural enemies can be effective in greenhouse but not in opened field. Entomopathogenic nematodes have been reported to be very efficient but their efficacy requires certain conditions like higher constant humidity levels.

All these setbacks aforementioned have triggered the research for environmental-friendly alternatives

The use of entomopathogenic fungi has emerged as an alternative to pesticides. The use of predatory mites and parasitoids can be effective in greenhouse but not in opened field. Entomopathogenic nematodes have been reported to be very efficient but their efficacy requires certain conditions like constant humidity.

All these setbacks aforementioned have triggered the research for environmental-friendly alternatives (Ekesi *et al.*, 1998).

Due to their ability to invade the insect through the cuticle, ease of production, formulation and application (Fargues, 1984; Butt and Goettel, 2000), the use of entomopathogenic fungi for the control of thrips has been investigated by many authors (Ekesi *et al*., 2000; Vestergaard, 1995; Butt and Brownbridge, 1997; Bradley *et al.*, 1998). In Kenya an isolate of *Metarhizium anisopliae* (Metcshnikoff) Sorokin has been investigated for the control of adult stages of *Megalurothrips sjostedti*, *Thrips tabaci* and *F. occidentalis*. (Ekesi *et al.*, 1998; Maniania *et al*., 2003; Maniania *et al*., 2002). However, it has been reported that larval stages of *F. occidentalis* are more resistant to fungal infection than the adult stage (Maniania *et al*., 2002; Vestergaard, 1995; Ugine *et al.*, 2005; Butt and Goettel, 2000). Therefore selection of isolates infective against larval stage is crucial step in the development of entomopathogenic fungi based biopesticides.

Several host crops of thrips are attacked by various diseases and other pest such as mites, aphids, white flies *etc*. Management of the above pest and diseases often involved usage of

different types of fungicides and insecticides. Hence there is a need to clearly understand the compatibility of biocontrol agent with other integrated pest management tools, which is crucial for their success and integration in IPM strategies (Maniania *et al.,* 2008).

The effectiveness of the fungus and its adoption can be only enhanced if innovative, economic and effective application strategies are available. Inundative release is so far the only mode of application of the fungus for the control thrips in a cropping system. In this regard, autoinoculation devices have been successfully developed for the control of several pests like Tsetse fly, fruit fly and leaf miner. Since thrips including *F. occidentalis* are attracted to semiochemicals (Teulon *et al.*, 2010; Koschier, 2006), it may be possible to apply the autodissemination strategy, whereby insects that are attracted to a semiochemical-baited inoculation device are infected with the pathogen before they return to the environment where they can disseminate the pathogen among host populations (Vega *et al.,* 2007). These autoinoculation devices need to be adapted for the management of thrips taking into consideration their behavior and ecology.

Plants are known to produce antifungal compounds that insect may sequester to resistant fungal infection (Poprawski *et al.,* 2000). Host plants variability affects tremendously the susceptibility of insect pests to entomopathogenic fungi (Ugine *et al*., 2006). For instance, it has been shown that within plant species, different varieties release volatiles that might inhibit fungal growth and therefore affect the success of fungal application (Ekesi *et al*., 2000). Food quality in relation to insect optimum development and fitness can reduce significantly the susceptibility of insect pest to *M. anisopliae* (Migiro *et al.*, 2011). As such, there is a need to evaluate the influence of commonly grown on the infectivity of the entomopathogenic fungi.

Considering all these facts, this study aimed to address some of the above constraints and research needs in the development of the fungal based product for the control of *F. occidentalis.*

1.2 Justification

Frankliniella occidentalis is a pest to diverse cultivated crops causing economical losses. Synthetic chemical pesticides are the most common control methods. Other control strategies such as cultural practice and plant sanitation are used in small scale with limited efficacy. The introduction of the maximum residue limit (MRL) by importing countries and the development of thrips resistance to synthetic chemicals pesticides underlie the research for environmental-friendly alternatives. Among the alternatives, entomopathogenic fungi hold potential because of their proven efficacy in the field. However, larval stages of *F. occidentalis* have been reported to be less susceptible to fungal infection compared to adult stage of thrips. There is the need therefore to identify isolates that are virulent to larvae. Since the use of fungus-based biopesticide represents only one component of IPM, its compatibility with other components such as agrochemicals used in crop production and tritrophic interaction between pathogen/host/host-plant has to be taken into consideration. Application strategy to deliver the pathogen is also an important aspect in the development of a biopesticide.

1.3 Hypotheses

i. Isolates of *Metarhizium anisopliae* and *Beauveria bassiana* are not pathogenic to larval stages of *F. occidentalis.*

ii. Fungal isolates are not genetically different.

iii. Agrochemicals do not have effect on growth parameter of entomopathogenic fungus and its virulence against *F. occidentalis.*

iv. Host-plant variations do not affect fungal virulence against *F. occidentalis.*

v. Application techniques of a fungal biocontrol agent are not effective in the management of thrips in greenhouse.

vi. Infection by fungal pathogen does not affect thrips vector competence.

1.4 Objectives

1.4.1 General objectives

The general objective is to develop a fungal based-product for management of the western flower thrips *F. occidentalis.*

1.4.2 Specific objectives

i. Screening of fungal isolates for selection of virulent isolates against larval stages of *F. occidentalis.*

ii. Evaluate the compatibility of the entomopathogenic with commonly used agrochemicals used in French bean production in Kenya.

iii. Develop a sustainable cost-effective application technique of the fungus for the management of *F. occidentalis* in screenhouse.

iv. Investigate interspecific and intraspecific effect of host plant on the virulence of fungus.

v. Investigate the effects of fungal infection on thrips vector competence of tospovirus.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Thrips classification

Thrips belong to the class Insecta in the Order Thysanoptera which include Terebrantia and Tubiliferans. The number of thrips is estimated to 7000 thrips of which 5500 are identified and confirmed (Lewis, 1997a). The genus *Frankliniella* belongs to the sub-order Terebrantia and family Thripidae. About 180 species are recognized in the genus *Frankliniella* mostly from the New World. *Frankliniella occidentalis* Pergande is the most destructive thrips in this genus which *Frankliniella occidentalis* is a Terebrantia from the Thripidae family. About 180 species are recognized in the genus *Frankliniella* mostly from the New World. For their accurate identification new tools such as the Lucid dichotomic key which is based on morphological and molecular characteristic of thrips are being developed (Moritz *et al*., 2001; EPPO, 2002).

Plate 2. 1: *Frankliniella occidentalis* life stages from egg to adult © Haas, Subramanian and Moritz.

Frankliniella occidentalis originates from western USA and has spread, since 1970, into many countries in Asia, Africa, Central and South America, Europe and Oceania. In northern European countries, it is a pest mainly in glasshouses, but in southern regions like in Kenya, it is a field pest, found even on fruit trees (EPPO, 2002; Lewis, 1997b; Kirk and Terry, 2003)

2.2 Biology of the western flower thrips *F. occidentalis*

Frankliniella occidentalis has six developmental stages (Plate 2.1), which typically occur in different plant parts (Lewis, 1997b). They lay eggs in leaf and flower tissue, and also in the fruits of vegetable crops (e.g. bean pods, capsicum fruits). Larva I and II thrive on leaves, in buds and flowers and at the base of some vegetable fruits. The prepupa and pupal stages occur in the soil or in hidden plant parts such as the bases of leaves. Adults are found on leaves, in buds and flowers. Adult *F. occidentalis* are less than 2mm long. Male adults are light yellow and have narrow abdomens, while female adults are larger than the males and vary in color from light yellow, yellow with brown splotches, to dark brown. They have slender bodies with two sets of narrow, clear, nearly veinless wings that have dark hairy, fringes (EPPO, 2002).

Female *F. occidentalis* adults live up to 30 days and lay 2–10 eggs per day (Lublinkhof *et al.*, 1977). At 20 ºC, development from egg to adult takes approximately 19 days. Their eggs are inserted into soft plant tissues, including flowers, leaves, stems and fruit (Lewis, 1997b; Gitonga *et al*., 2002).

2.3 Economic Importance

The host range of *F. occidentalis* includes over 250 species of herbaceous and woody plants belonging to 62 families (EPPO, 2002; Cuthbertson *et al*., 2005; Lewis, 1997b).

Thrips infestation can cause white or brown spots on the leaves where the plant cells have been destroyed. These damages are not fair for commercial ornamental plants which are most of the time rejected by customers. This affects significantly reduces the quality and the export value of flowers in Kenya (Nderitu *et al*., 2007).

Damages on plants are various:

- Silvering on the abaxial side of the leaves;
- Blemishes on the flowers;
- Flower abortion and dropping in case of severe incidence;
- Blemishes on the growing beans and their malformation in legumes.

Western flower thrips *F. occidentalis* is also an efficient vector of tospovirus especially tomato spotted wilt virus during feeding (Mound, 2001; Baker *et al*., 1993; Lewis, 1997b). Tospovirus can infect plants belonging to 35families including the Solanaceae, Asteraceae, Leguminaceae, Brassicaceae, and Bromiliaceae (Mound, 2001; Tsuda *et al*., 1996; Ullman *et al*., 1995; Whitfield *et al*., 2005; Lewis, 1997b). Plants infected with tospoviruses are often mistaken for fungal and bacterial infection by tospovirus all infection by fungi and bacteria mislead farmers who resort to application of fungicides without any tangible benefit. To date there is no efficient method for the management of tospovirus epizootics. The use of resistant cultivars, plant sanitation and chemical pesticides are often recommended to farmers (Groves *et al*., 2001; Aramburu *et al*., 2000; Bennison *et al*., 2001).

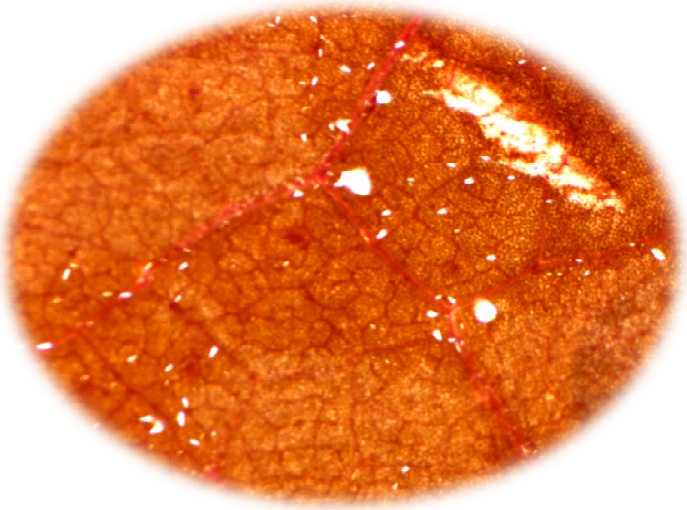

Plate 2. 2 : Feeding punctures of *Frankliniella occidentalis* on a French bean leaf © Nyasani et al.

2.4 Control of *Frankliniella occidentalis*

Frankliniella occidentalis is usually controlled in commercial greenhouses using different methods like prevention of infestation through management options like synthetic pesticide application, intercropping, mulching, cultural practice (managing crop plants, crop plant wastes, weeds, soil, and environmental conditions to eliminate WFT), biological control (using predatory insects and entomopathogenic fungi).

2.4.1 Chemical control

Chemicals are the most widely used method of thrips control (Lewis, 1997a). Diverse pesticides like chlorpyrifos-methyl, methiocarb, methamidophos, acrinathrin, endosulfan, deltamethrin and formetanate are used to control thrips (Rachappa *et al.*, 2007). The most likely reason for thrips resistance to chemical pesticides could be explained by the fact that intensive insecticide used in horticulture in 1970s and 1980s has selected an insecticide resistant strain or strains of WFT. These strains of *F. occidentalis* have then established in glasshouses across North America and spread from there to Europe, Asia, Africa and Australia. The development of pest resistance to insecticides necessitates higher doses and more frequent pesticide applications resulting in harmful side effects on the environment and non-target species (Jensen, 2004; Broughton and Heron, 2009) Therefore, combinations of synthetic chemicals with entomopathogenic fungi are often used to control the pest (Rachappa *et al*., 2007). Alternation of pesticides is often recommended to avoid development of pest resistance. However this cannot be applied by most of farmers and smallholders in Africa due to the high-costs of synthetic chemical pesticides.

2.4.2 Parasitoid and predatory insects

The use of predators and parasitoids is very common in biological control. Several species are identified as thrips parasitoids and are being studied for their integration in pest management (Loomans and Murrai, 1997; Ludwig and Oetting, 2001).

Parasitoids, *Ceranisus menes* (Hymenoptera: Eulophidae) is an effective parasitoid of thrips larval stages (Gitonga, 2008). *Megaphragma priesneri* Kryger, *M. mymaripenne* Timberlake, *Thripobius semiluteus* Boucek, *Pediobius thysanopterus* Burks, *Goetheana shakespearei* Girault are very common thrips parasitoids in Israel. *Megaphragma priesneri* Kryger, *M. mymaripenne* Timberlake, *Thripobius semiluteus* Boucek, *Pediobius thysanopterus* Burks, *Goetheana shakespearei* Girault are very common thrips parasitoids in Israel (Loomans, 2006; Kuslitzky, 2003).

Anthocorids bugs, particularly *Orius* species, are important predators of agricultural pests such as thrips, aphids and spider mites. *Orius laevigatus* Fieber; *O. strigicollis* Popoius, *O. albidipennis* Reuter, *O. niger* Wolff have been reported to be very effective control agents of thrips.

The mirid bug *Dicyphus tamaninii* Wagner is a polyphagous predator of greenhouse whitefly and western flower thrips on cucumber (Gabarra, 1995).

Predatory mites are also used against thrips; *Phytoseiulus persimilis* Athias-Henriot, *Typhlodromips montdorensis* Schicha are also used to control pest such as *Frankliniella schultzei* Trybom, *Aculops lycopersici* Massee, *Tetranychus urticae* Koch in Australia (Steiner *et al*., 2003). In the same way, *Amblyseius andersoni* Chant, *A. californicus* Mc

Gregor, *A. cucumeris* Oudemans and *A. degenerans* Berlese feed on *Tetranychus urticae* Koch and *F. occidentalis* larvae as prey (Jacobson *et al*., 2001b; Blaeser *et al*., 2002).

Under ample prey supply, those natural enemies, particularly *O. laevigatus* have high reproductive success on thrips; hence thrips represent a higher prey quality to the bugs (Zhang *et al*., 2006; Venzon *et al*., 2002; Funderburk, 2001).

Integrated Pest Management strategies should take into consideration these predators which play an important role in pest population control especially in greenhouse. However, in case of heavy outbreaks in field the impact of natural enemies of thrips is not significant therefore there is a need to look for other complementary methods by associating natural enemies with other IPM tools (Down *et al*., 2009; Ludwig and Oetting, 2001; Jacobson *et al*., 2001b).

2.4.3 Entomopathogenic fungi

The use of entomopathogenic fungi is very promising for thrips control (Butt and Brownbridge, 1997; Ekesi *et al*., 2000). Cuticular infection pathway is an advantage of entomopathogenic fungi as compared to *per os* infection pathways of other pathogens (Butt *et al*., 2001; Goettel and Inglis, 1997; Lacey, 1997). The amount of conidia reaching the target and the application time are very important parameters that should be standardized for an effective pest management (Ugine *et al*., 2007; Ugine *et al*., 2005).

2.4.3.1 *Metarhizium anisopliae* (Metschnikoff) Sorokin

Metarhizium anisopliae is one of the most utilized entomopathogenic fungi for pest control (Ugine, 2007 #82; Ugine, 2005 #113). The susceptibility of *F. occidentalis* to *M. anisopliae* has been reported by many workers (Vestergaard, 1995 #114; Maniania, 2002 #11). Conidia

germinate and produce appressoria on the host within 12 h post-inoculation. The fungus colonizes the insect hemocoel from day three and sporulates approximately six days post-inoculation (Vestergaard, 1995; Maniania *et al*., 2002). Application of *Metarhizium* can cause significant reduction of thrips population growth in the field (Ekesi *et al*., 2000; Maniania *et al*., 2002). The spray treatment of *Metarhizium sp*. strains can cause significant reduction of WFT population growth in the field (Ekesi *et al*., 2000; Maniania *et al.*, 2002).

Some authors reported synergistic effect between the entomopathogenic fungus *Metarhizium anisopliae* and synthetic insecticides, even though this processes is not clearly elucidated (Borgio *et al*., 2008; Depieri *et al*., 2005; Irigaray *et al*., 2003; Mohan *et al*., 2007; Hiromori, and Nishigaki, 2001; Dara and Hountondji, 2001; Alizadeh *et al*., 2007). Therefore, combinations of synthetic chemicals with entomopathogenic fungi are often recommended for the control of thrips (Shi *et al*., 2005; Neves *et al*., 2001).

2.4.3.2 Effects of *M. anisopliae* on non-target organisms

The safety of fungi against non-target organisms especially biological control agents and vertebrates especially humans needs to be considered at many levels. In considering safety towards all organisms, the National American Microbial working Group has pointed several issues: competitive displacement, allergenicity, toxigenicity, pathogenicity to non-target hosts (Inglis *et al*., 2001). Studies have compared to synthetic chemical insecticides and *M. anisopliae* application. It was shown that fungal treatment did not affect beneficial and non-target organisms as compared to chemical treatment while maintaining the population of thrips under control (Ekesi and Maniania, 2000a).

Studies carried out by CERES-Locutox had revealed possible contamination of *Metarhizium spp.* and *Beauvaria sp.* on non-target Hymenoptera parasitoids, i.e., *Bracon hebetor* and *Epidinocarsis lopezi* (Danfa and Van der Valk, 1999). That study was later contradicted as

the insects were stressed and the rates of conidia used were different from field doses (Stolz *et al*., 2002).

2.4.3.3 Mass production of *M. anisopliae*

A successful microbial insecticide should have stable virulence and should be amenable for mass production, formulation and application (Chandler *et al.,* 2008; Navon and Aschers, 2000). Conidia are probably the most appropriate propagule for field use due to their environmental stability under adverse weather conditions as compared to application of hyphaes or blastospores (Leland and Behle, 2004; Jenkins et al., 1998; Soper and Ward, 1981). Mass production using rice as a substrate is the most commonly used method.

2.4.3.4 Application strategies

Understanding the behavior of a pest is a relevant step in efficient control strategy design. *Frankliniella occidentalis* is a flower dwelling thrips and prefers secluded and concealed parts of plants and also because of frequent molting; larval stages of *F. occidentalis* are less susceptible to fungal application (Vestergaard, 1995; Maniania *et al*., 2002; Ugine *et al*., 2007).

Previous studies have shown that the application method may not affect the efficacy of the fungus (Ugine *et al*., 2005), but environmental conditions in field and many other factors like watering, application time, formulation and host/plant/pathogen interactions should be considered during development biological control strategy for a pest (Boissot *et al*., 1998; Ugine *et al*., 2007; Alghali *et al,* 1991; Morsello, 2007; Trdan *et al*., 2003). As such, timely application could affect the efficacy of fungal biopesticides (Ugine *et al*., 2007), spray manipulation and the frequency of spray could affect thrips populations (Ugine *et al*., 2007).

Therefore the development of an efficient application to improve fungal efficacy is of paramount important in biological control.

2.4.4 The use of attractants in combination with sticky traps

Understanding the behavior of a pest is a relevant step in efficient control strategy design. Thrips are attracted to a certain range of colors and odor which are therefore currently used for thrips monitoring and control (Ludwig and Oetting, 2002; Kirk and Hamilton, 2004; Murai *et al.*, 2000).

Recent studies carried out at *icipe* have demonstrated the potential of blue color sticky cards in attracting exclusively *F. occidentalis* and less attractive to the natural enemies as compared to the yellow sticky cards (Chen *et al*., 2004; Subramanian *et al*., 2009). Various compounds such as pyridins, aldehydes (Teulon *et al*., 2010) have also been reported to attract thrips species (El-Sayed *et al.*, 2009; Davidson *et al*., 2007) and are being considered for the control of thrips. Hence, this informations can be used in an autoinoculation system where thrips are attracted to a device with semiochemical to infect thrips populations for effective and sustainable pest control (Vega *et al*., 2007).

CHAPTER THREE

1.0 GENERAL MATERIALS AND METHODS

3.1 Study site

The research work was conducted at *icipe'*s Headquarters, Duduville, Nairobi. Laboratory work was undertaken in the Arthropod Pathology Unit (APU) at *icipe.*

3.2 Thrips colony rearing

Thrips colonies were reared at Animal Rearing and Quarantine Unit, *icipe*. A colony of *F. occidentalis* from field collected insects in ventilated plastic containers (16 X 16 X 6cm) with thrips-proof nets and filter paper in cover. French bean *Phaseolus vulgaris* (L) pods collected from the greenhouse and surface sterilized with sodium hypochlorite and rinsed with sterile water were used for rearing.

Plate 3. 1: Rearing containers for rearing *Frankliniella occidentalis* in the laboratory, *icipe*, Duduville

Adults of thrips were allowed to lay eggs on the pods; three days after pods containing eggs were removed and placed in another clean container. Filter paper and paper towel were placed below the pods in the container to facilitate pupation (Loomans and Murai, 1997). Filter paper and paper towel with pupae were maintained for adult emergence and these adults were subsequently used for furthering the generations. Larval instars were identified based on the number of days after oviposition and the size of the individuals.

3.3. Cultures of entomopathogenic fungi

Fungal isolates were selected from the *icipe*'s Arthropod Germplasm. They were cultured on Sabouraud Dextrose Agar (SDA) in 9-cm Petri dishes and incubated at 25 ± 2°C in complete darkness.

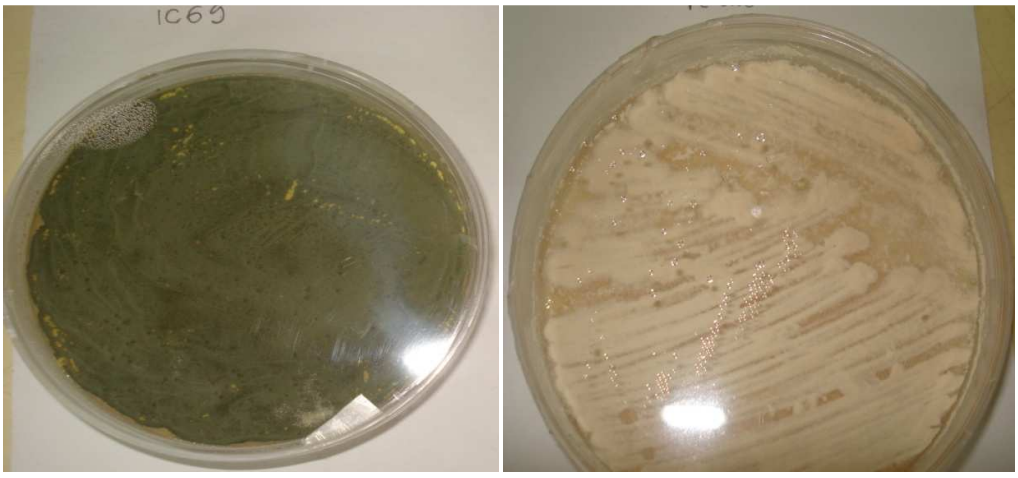

Plate 3. 2: *Metarhizium anisopliae* and *Beauveria bassiana* plates cultured on Sabouraud Dextrose Agar (SDA)

3.3.1 Preparation of inoculums

Conidia were harvested by scrapping the surface using a spatula and suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads. Conidial suspensions were vortexed for 5 minutes to produce a homogenous suspension. Conidial concentrations were determined using a haemocytometer.

Plate 3. 3: Universal bottles containing 10 ml suspension of *Metarhizium anisopliae* and *Beauveria bassiana*

Bottles were vortexed for five minutes to produce homogenous conidial suspension. Conidial concentrations were determined by using a hemacytometer and the desired concentration was obtained by serial dilutions.

3.3.2 Viability of the inoculum

The viability of conidia was determined before any bioassay by spread-plating 0.1 ml of a 3×10^6 conidia ml^{-1} suspension onto 9-cm Petri dishes containing SDA medium. A sterile microscope cover slip was placed on each plate, and the plates were incubated in complete darkness at 25 ± 2°C for 16h (Lacey, 1997). The average percentage of and examined after 20 h. Percentage germination of conidia was determined by assessing the number of germ

tubes formed among 100 random conidia on the surface area covered by each cover slip under the light microscope (400 ×). Four replicate plates of the isolates were

3.3.3 Mass production of *Metarhizium anisopliae*

Metarhizium anisopliae isolate ICIPE 69 was used in this study and its virulence against *F. occidentalis* has been previously established (Maniania *et al*., 2002). Conidia were mass-produced on whole rice substrate in Milner bags (60 cm long by 35 cm wide).

Plate 3. 4: Description of mass production procedures of *Metarhizium anisopliae* conidia on rice

The rice was autoclaved for 1 h at 121°C and inoculated with a 3-days-old culture of blastospores (Jenkins *et al*., 1998). This was then incubated for 21 days at 20-26 ºC, 40-70% RH. The rice containing fungal spores was then allowed to dry for 5 days at room temperature. Conidia were harvested by sifting the substrate through a sieve (295- μm mesh size) and stored at 4-6 °C until used. At harvest percent viability was at 92 % on SDA plates.

3.3.4 Methods of inoculation

A Burgerjon spray tower (Burgerjon, 1956) was used to spray conidial inocula either on substrates (bean pods) or directly on insects. In the control treatments, with sterile distilled water containing 0.05 % Triton X-100 was used.

Autoinoculation device similar to the one used by Migiro *et al*. (2010) was also used to infect *F. occidentalis* in field-cage. Briefly, Lynfield trap (11 cm diameter × 10 cm height) was perforated with five entry/exit holes (2 × 3 cm) made near the top and bottom of the bottle at alternate positions. A velvet (8 cm diameter × 8.5 cm length) and blue netting (3.5 cm diameter × 11 cm height) wrapped around a smaller inner cylindrical bottle (5.2 cm diameter and 6 cm in height) that was then hung in the trap. In addition, a 9.3-cm disc (from blue sticky card) was added on the top and bottom of the device to increase the attraction (Chen *et al.*, 2004). The semiochemical dispenser was inserted inside the smaller plastic diameter bottle.

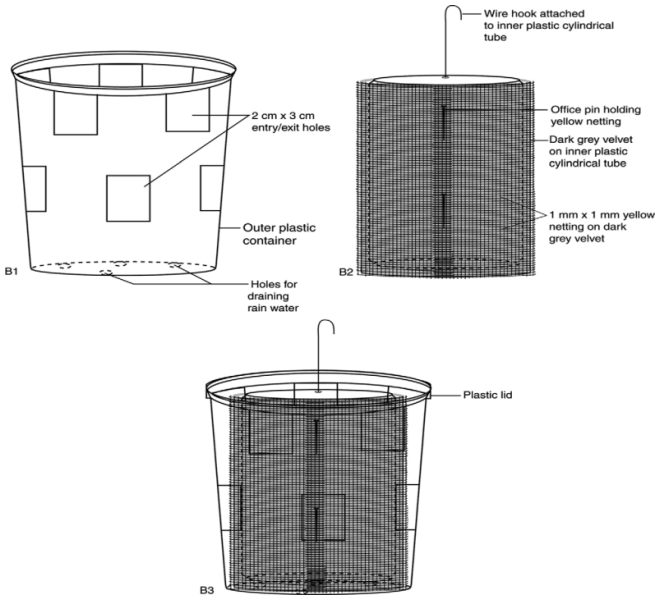

Figure 3. 1: Description of an autoinoculative device made from a modified Lynfield trap (Migiro *et al.*, 2010)

Plate 3. 5: Burgerjon spray tower

CHAPTER FOUR

4.0 SELECTION OF FUNGAL BIOLOGICAL CONTROL AGENT OF THE WESTERN FLOWER THRIPS *FRANKLINIELLA OCCIDENTALIS* (PERGANDE)

4.1 Introduction

The western flower thrips (WFT), *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) is a vicious quarantine pest of horticultural crops worldwide (EPPO, 2002; Lewis, 1997b). In addition to crop damage such as abscission of buds, abortion of flowers, and malformation of pods, WFT are efficient vectors of tospoviruses (Lewis, 1997b). Synthetic chemical pesticides are widely used for control of thrips, despite their toxicity and hazardous effects to humans and the environment (Nderitu *et al*., 2008). In addition, the WFT have developed resistance to major groups of synthetic chemicals (Broughton and Herron, 2009; Jensen, 2004). There is, therefore, the prevailing need to develop ecologically sound and sustainable alternative for management of thrips. Entomopathogenic fungi are among the control strategies being developed (Ekesi and Maniania, 2000a; Ekesi and Maniania, 2007). For instance, Maniania *et al*. (2002) reported that application of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) significantly reduced WFT in chrysanthemum crop; but the control of larval populations was much lower than for adults. Similar observations were made in laboratory bioassays by Vestergard, (1995) and Ugine *et al*., (2005) with *M. anisopliae* and *Beauveria bassiana* (Balsmo) Vuillemin. The objective of the present study was, therefore, to screen different fungal isolates of *M. anisopliae* and *B. bassiana* for selection of virulent isolate(s) against second-instar larval stage of WFT. Other parameters such as conidial production and evolutionary phylogenetic variability among the fungal isolates were also considered using molecular tools.

4.2 Materials and methods

4.2.1 *Frankliniella occidentalis* colony

Insects were obtained from the Animal Rearing and Quarantine Unit at the International Centre of Insect Physiology and Ecology (*icipe*). They were reared on French bean pods *Phaseolus vulgaris* (L) var. *Samantha* at 25 ± 2 ºC, 60-80% RH, with a 12 L: 12 D photoperiod as detailed in Section 3.2. Second-instar larval stage was used in the experiments.

4.2.2 Fungal isolates

Fungal isolates were selected from *icipe*'s Arthropod Germplasm Centre (Table 4.1). Fungal cultures and viability is detailed in section 3.3.

Table 4. 1: List of fungal isolates and their origin tested against second-instar larvae of *Frankliniella occidentalis* and percentage of germination after 16h on SDA plates at 25 ± 1 ºC.

Fungal species	Isolates	Locality (Country)	Source	% Germination
Metarhizium	ICIPE 7	Rusinga Island (Kenya)	*Amblyoma variegatum*	92.0 ± 1.6
anisopliae	ICIPE 18	Mbita (Kenya)	Soil	92.8 ± 1.6
	ICIPE 20	Migori-Kenya	Soil	96.5 ± 0.8
	ICIPE 30	Kendu Bay (Kenya)	*Busseola fusca*	89.4 ± 1.2
	ICIPE 41	Migori (Kenya)	Soil	100
	ICIPE 69	Matete (DRC)	Soil	93.5 ± 0.6
	ICIPE 78	Ungoye (Kenya)	*Temnoschoita nigroplagiata*	90.7 ± 1.0
	ICIPE 84	(Senegal)	*Ornitacris turbida*	100
	ICIPE 625	Kabuti (Kenya)	Soil	100

	ICIPE 665	Ahero Plains (Kenya)	Soil	92.8 ± 1.3
Beauveria bassiana	ICIPE 279	Kericho (Kenya)	Coleopteran larvae	97.0 ± 0.7
	ICIPE 284	Mauritius	Soil	95.0 ± 0.7
	ICIPE 620	Kapsorok (Kenya)	Soil	100
	ICIPE 621	Motinet (Kenya)	Soil	100
	ICIPE 622	Kapiti sondu (Kenya)	Soil	100
	ICIPE 646	(Mauritius)	Soil	96.8 ± 0.9
	ICIPE 659	Kapmonyok (Kenya)	Soil	100
	ICIPE 664	Bungoma (Kenya)	Soil	100

4.2.3 Screening of *Metarhizium anisopliae* and *Beauveria bassiana* isolates for time mortality

Ten (10) ml of standard concentration of 1×10^7 conidia ml^{-1} was sprayed on four pods of French beans using a Burgerjon spray tower (Burgerjon, 1956). Pods were allowed drying for 5 min, after which they were transferred to 30-ml glass tubes. Twenty (20) 2^{nd}-instard larvae of WFT were then introduced per tube. In the control treatments, pods were sprayed with sterile distilled water containing 0.05 % Triton X-100. Mortality was recorded daily for 10 days. Dead insects were placed in humidified chamber to allow the development of mycosis on the surface of cadaver. Each treatment consisted of four replicates of 20 insects each and was repeated three times.

4.2.3 Dose-mortality assays

Dose-mortality relationships were calculated for the most pathogenic isolates by using five concentration of inoculum: 3×10^6; 1×10^7; 3×10^7; 1×10^8 and 3×10^8 conidia ml^{-1}. Test-

larvae were incubated at 25 ± 2 ℃ and 90 ± 2 % RH with a photoperiod of 12 h light: 12 h dark. Mortality was recorded daily for 10 days. Dead thrips were placed in humidified chamber to allow the development of mycosis on the surface of cadaver. Each treatment consisted of four replicates of 20 thrips each and was repeated three times.

4.2.4 Conidial production on *Frankliniella occidentalis* larvae

Three fungal isolates with lowest LC_{50} values were compared for conidial production. Second-instar larvae of WFT were exposed for 24 h to fungus-treated French bean pods at the concentration of 1×10^8 conidia ml^{-1}, after which they were transferred onto sterile containers containing clean pods. At 3, 6 and 9 days post-infection, five mycosed thrips were collected dried in an oven for 30 minutes at 30 ± 1 ºC and transferred individually into 2-ml cryogenic tubes containing 0.1 ml of sterile 0.05 % Triton X-100. The tube was then vortexed for 5 min to dislodge conidia from the insect body and the number of conidia was determined using a haemocytometer (Hausser, Scientific Horsham, USA). The experiment was repeated four times.

4.2.5 Characterization of fungal isolates based on Internally Transcribed Spacer sequences.

4.2.5.1 DNA extraction from fungal isolates

Pure cultures of *M. anisopliae* isolates ICIPE 7, ICIPE 20 and ICIPE 69 were produced on SDA. Equal amounts (0.1g) of conidia of each of the isolates were weighed in microcentrifuge tubes on a weighing balance (Mettler AT 261 Delta, Listers 2000). DNA was extracted using a slight modification of the CTAB method described by Doyle and Doyle

(Doyle and Doyle, 1990) and resuspended in pre-warmed sterile deionized water. The primer pairs n-SSU-1766-5 (ITS5) and nu-LSU-0041-3 (ITS4) (White *et al.*, 1990) were used to amplify the ITS sequences of the genomic DNA. PCR amplification reactions were carried out in a total volume of 20µl containing PCR buffer (Genscript, Piscataway, NJ, USA), 2.5mmol l^{-1} of each dNTP (Genscript), 0.2µl of each primer, 2.5mmol l^{-1} of $MgCl_2$, 0.5 units *Taq* DNA polymerase (Genscript) and ~25ng of genomic DNA. PCR amplification conditions involved initial denaturation at 94 °C for 3min, followed by 30 cycles of 94 °C for 40s, annealing temperature of 52 °C for 40s with an extension at 72 °C for 1min and final elongation at 72 °C for 10min. These reactions were carried out on a PTC-100 thermocycler (MJR Inc., Minneapolis, MN, USA). Negative controls without fungal DNA were run for each experiment to check for contamination of reagents.

4.2.5.2 DNA quantification and sequencing

The amplification products were separated by electrophoresis in 1% agarose gels containing ethidium bromide (3µl), in 1 × TAE buffer for 1h at 70Vcm^{-1}. DNA was visualized under UV light, and recorded using a Kodak Gel imaging system (Gel logic 200, Carestream Health, New Haven, CT, USA). The lengths of the amplicon products were estimated by comparison with 1kb Smart DNA ladder (Noxo, Tallinn, Estonia). The PCR products were purified using QuickClean DNA gel extraction kit (Genscript) and sequenced at the International Livestock Research Institute (ILRI).

4.2.6 Data analysis

Percent mortality was corrected for control mortality (Abbott, 1925) and normalized by arcsine transformation before being subjected to analysis of variance (ANOVA) using PROC

GLM, at 95% level of significance. Student-Newman-Keuls analysis was used to separate the means as a post-ANOVA procedure. Median lethal time (LT_{50}) and median lethal concentration (LC_{50}) were estimated using logistic regression. These analyses were carried out using GENMOD procedure of SAS version 9.2. 95% confidence intervals were used to identify significant differences among the values of LT_{50} and LC_{50}. A Pearson correlation analysis was carried out to relate mortality rate with the conidial production.

The ITS sequences of the PCR products were edited using Bioedit (version7.0.5.3) (Hall, 1999) and aligned using Clustal W (version 2.012) (Larkin *et al*., 2001) software.

A Basic Local Alignment Search tool (BLAST) was performed using NCBI, EMBL and Fungal Genome Search databases. The first best hit accession number was considered.

4.3 Results

In viability tests, conidial germination varied between 89 and 100% (Table 4. 1). Mortality in the controls was low and did not exceed 15% in all the experiments.

Table 4. 2: Virulence of fungal isolates against second-instar larvae *Frankliniella occidentalis*: Percent mortality and LT_{50} values at the concentration of 10^7 conidia ml^{-1} 10 days post-treatment.

Fungal species	Isolates	Mortality (% ± SE)*	LT_{50} (days) (95% CI)**	Slope (± SE)
Metarhizium anisopliae	ICIPE 20	56.2 ± 2.9a	8.5 (8.3 - 8.8)	4.7 ± 0.1
	ICIPE 69	55.9 ± 1.9a	8.2 (8.0 - 8.4)	4.1 ± 0.1
	ICIPE 7	51.2 ± 5.0ab	8.3 (8.0 - 8.5)	4.0 ± 0.1
	ICIPE 665	49.6 ± 3.0ab	8.4 (8.1 - 8.7)	3.7 ± 0.1
	ICIPE 18	44.1 ± 3.0abc	10.6 (10.1 - 11.2)	2.8 ± 0.1
	ICIPE 41	48.2 ± 3.5abc	9.2 (9.0 - 9.6)	4.0 ± 0.1
	ICIPE 625	48.3 ± 3.5abc	10.5 (10.1 - 10.6)	4.3 ± 0.1
	ICIPE 84	43.3 ± 2.5abc	11.3 (10.8 - 11.9)	3.5 ± 0.1
	ICIPE 30	40.4 ± 4.0bcd	11.8 (11.2 - 12.4)	3.7 ± 0.1
	ICIPE 78	40.9 ± 3.2bcd	11.1 (10.7 - 11.7)	3.6 ± 0.1
Beauveria bassiana	ICIPE 620	45.9 ± 2.1abc	10.2 (10.0 - 10.6)	4.7 ± 0.1
	ICIPE 621	44.6 ± 2.5abc	11.6 (11.1 - 12.2)	4.1 ± 0.1
	ICIPE 646	42.5 ± 2.5bc	10.5 (10.0 - 11.0)	3.4 ± 0.1
	ICIPE 659	38.0 ± 2.3bcd	12.9 (12.2-13.7)	3.9 ± 0.1
	ICIPE 284	35.0 ± 4.2cd	14.8 (13.9 - 16.0)	3.5 ± 0.1
	ICIPE 279	29.9 ± 2.8de	17.7 (16.2 - 19.6)	2.6 ± 0.1
	ICIPE 664	24.6 ± 2.0e	24.8 (21.9 - 28.7)	2.7 ± 0.1
	ICIPE 622	23.8±1.4e	33.0 (27.8 - 40.7)	2.0 ± 0.1

* Within column means followed by the same letters are not significantly different by Student-Newman-Keuls ($P < 0.05$). ** Within column LT_{50} values with overlapping 95% CI are not significantly different

All tested fungal isolates were pathogenic to the second-instar WFT (Plate 4.1) at a concentration of 1×10^7 conidia ml^{-1}, causing mortalities of between 24 and 56% (Table 4.2). *Metarhizium anisopliae* isolates ICIPE 20 and ICIPE 69 caused the highest mortality and it was significantly different from other *M. anisopliae* isolates ICIPE 30, and 78 and *B. bassiana* isolates ICIPE 646, 659, 284, 279, 664, and 622 ($F_{17,195} = 17.37$, $P < 0.001$). *Beauveria bassiana* isolates ICIPE 664 and ICIPE 622 caused the lowest mortalities. Other *M. anisopliae* isolates ICIPE 7, 665, 18, 41, 625 and 78 and *B. Bassiana* isolates ICIPE 620

and 621 were not significantly different from ICIPE 20 and 69 in term of mortality (Table 4. 2). *Metarhizium anisopliae* isolates ICIPE 7, ICIPE 20, ICIPE 69 and ICIPE 665 had the shortest LT_{50} values causing mortalities within 8.2 - 8.4 days as compared to the other fungal isolates (Table 4. 2).

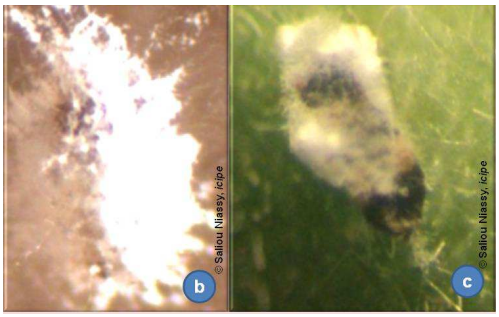

Plate 4. 1: Mycosed larvae of *Frankliniella occidentalis* infected with standard concentrations of *Beauveria bassiana* (left) and *Metarhizium anisopliae* (right)

Among the seven isolates of *M. anisopliae* and one isolate of *B. bassiana* selected for lethal concentration response bioassays, *M. anisopliae* isolate ICIPE 69 had the lowest LC_{50} value followed by ICIPE 7 and ICIPE 20 (Table 4.3). *Metarhizium anisopliae* isolate ICIPE 69 produced significantly more conidia than the other two isolates in all the three sampling dates ($F_{2, 31} = 8.9$, $P < 0.0009$) (Figure 1).

Table 4. 3: Lethal concentration values (LC_{50}) of selected fungal isolates against second-instar larvae of *F. occidentalis.*

Species	Isolates	LC_{50} (95% CI) ($\times 10^8$ conidia ml^{-1})	Slope (± SE)
Metarhizium anisopliae	ICIPE 69	0.1 (0.0 - 0.1)	2.1 ± 0.1
	ICIPE 7	0.2 (0.1 - 0.2)	1.2 ± 0.0
	ICIPE 20	0.3 (0.2 - 0.3)	1.1 ± 0.0
	ICIPE 41	0.8 (0.6 - 1.0)	0.9 ± 0.0
	ICIPE 84	3.6 (2.5 - 5.8)	1.0 ± 0.0
	ICIPE 18	18 (8.1 - 58.0)	0.7 ± 0.0
	ICIPE 625	4.0 (2.8 - 6.4)	1.0 ± 0.8
Beauveria bassiana	ICIPE 620	14.4 (7.5 -36.6)	0.8 ± 0.1

Conidia production also significantly increased over the sampling days, 3, 6 and 9 days ($F_{2,31}$ = 18.9, P < 0.0001, SNK) post-infection (Figure 4.2). A correlation between conidial production and mortality (Pearson R = 0.65, P < 0.001) was observed (Table 4.4).

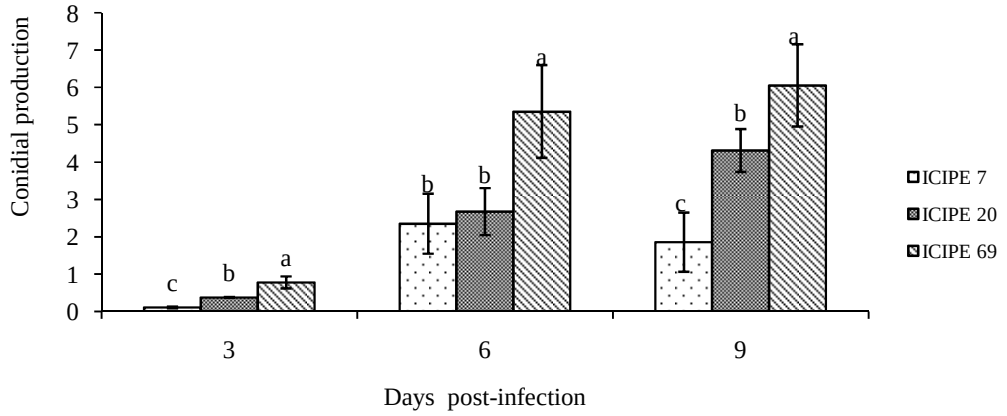

Figure 4. 1: Mean conidial production ($\times 10^5$ conidia) of three isolates of *Metarhizium anisopliae* following infection of second-instar larvae of *Frankliniella occidentalis*.

Table 4. 4: Correlation between mortality and conidial production of three *Metarhizium anisopliae* isolates ICIPE 7, ICIPE 20 and ICIPE 69 applied on second-instar larvae of *Frankliniella occidentalis* at 1×10^8 conidia ml^{-1}.

Isolates	Mean mortality (%)	Mean conidial production ($\times 10^5$ conidia)
ICIPE 7	55.5 ± 6.7	2.5 ± 0.9
ICIPE 20	50.8 ± 7.6	3.4 ± 1.2
ICIPE 69	70.9 ± 10.5	6.9 ± 2
Pearson	R = 0.65; P < 0.0001 ; N = 36	

ITS fragment (Plate 4.2) of ICIPE 7, ICIPE 20 and ICIPE 69 were sequenced and aligned using Clustal W (version 2.012). This indicated two base differences in ICIPE 69 as compared to ICIPE 7 and ICIPE 20 (Fig 4.2).

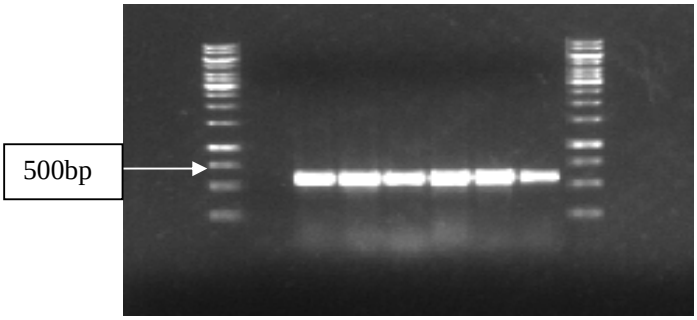

Plate 4. 2: PCR products electrophoresed through 1% EtBr-stained agarose gel from left to right on Lane 1 Marker-1Kb DNA ladder, 2., -ve control, 3. ICIPE 7,4. ICIPE 20, 5. ICIPE 41, 6. ICIPE 69, 7. ICIPE 78, 8. ICIPE 655, 9. Marker-1Kb.

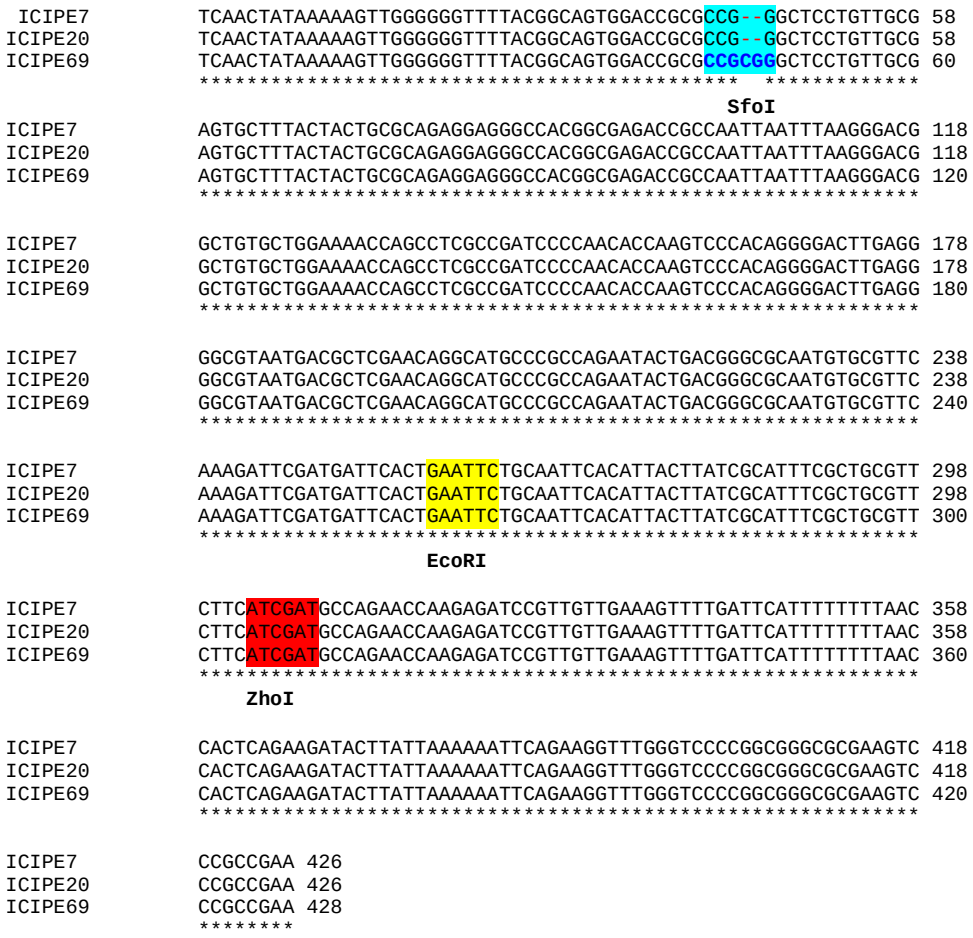

Figure 4. 2: ITS4-ITS5 sequence alignments of DNA of three *Metarhizium anisopliae* isolates ICIPE 7, ICIPE 20 and ICIPE 69 showing the restriction sites EcoR1, ZhoI and SfoI. Sections of the two sequences marked and unmarked with asterisks indicate homology and divergence, respectively, between the sequences.

The sequence CCGCGG in ICIPE 69 which includes the two base pair difference from the other isolates corresponds to restriction site sequence *Serratia fonticola* I (SfoI). Restriction sites were also identified on the ITS sequence EcoRI and ZhoI and were common for all the three *M. anisopliae* isolates.

A Basic Local Alignment Search Tool on NCBI, EMBL and Fungal Genome Search indicated a low expect value(E) and similarity values ranging between 97 and 100% with *M. anisopliae* FJ545302, FJ609312 respectively on NCBI and EMBL. The Fungal Genome Search database identified the isolates as affiliated to *M. anisopliae* var. *anisopliae* AF136376 (Table 4.5).

Table 4. 5 : Basic Local Alignment Search Tool of *Metarhizium anisopliae* ICIPE 7, ICIPE 20 and ICIPE 69 ITS4, ITS5 sequences using NCBI, EMBL and Fungal Genome Search databases

Isolates	Length (bp)	Best hit	E value (E)	Identity (%)	Species
			NCBI		
ICIPE 7	426	FJ545302	0.0	100	*M. anisopliae*
ICIPE 20	426	FJ545302	0.0	100	*M. anisopliae*
ICIPE 69	428	FJ545302	0.0	99	*M. anisopliae*
			EMBL		
ICIPE 7	426	FJ609312	$4.3\,e^{-85}$	98	*M. anisopliae*
ICIPE 20	426	FJ609312	$5.3\,e^{-86}$	98	*M. anisopliae*
ICIPE 69	428	FJ609312	$5.3\,e^{-86}$	97	*M. anisopliae*
			Fungal Genome Search		
ICIPE 7	426	AF136376	$6.6\,e^{-87}$		*M. anisopliae* var. *anisopliae*
ICIPE 20	426	AF136376	$6.6\,e^{-87}$		*M. anisopliae* var. *anisopliae*
ICIPE 69	428	AF136376	$5.3\,e^{-86}$		*M. anisopliae* var. *anisopliae*

4.4 Discussion

The aim of this study was to identify potential fungal candidate(s) for control of the larval stage of *F. occidentalis* that has been reported to be refractory to fungal infection (Vestergaard, 1995; Maniania *et al*., 2002; Ugine *et al*., 2005) and to further characterise these isolates based on the ITS gene sequence (Freed *et al*, 2011). At the concentration of 1×10^7 conidia ml^{-1}, all the fungal isolates tested were pathogenic to the second-instar larvae of WFT; however mortality and LT_{50} values varied between the isolates. Such variations have already been reported for fungal pathogens in many groups of insects (Migiro *et al*., 2010; Mburu *et al*., 2009; Ekesi *et al*., 1998; Dimbi *et al*., 2003). Four isolates of *M. anisopliae* (ICIPE 20, ICIPE 69, ICIPE 7 and ICIPE 665) with LT_{50} between 8.0 and 8.8 days outperformed the other fungal isolates (Table 4. 2).

When seven isolates of *M. anisopliae* and one of *B. bassiana* were challenged for the LC_{50} bioassays, only three isolates of *M. anisopliae* (ICIPE 7, ICIPE 20 and ICIPE 69) had the lowest LC_{50} ($1\text{-}3 \times 10^7$ conidia ml^{-1}) (Table 4. 3). Virulence has always been one of the most important parameters considered for strain selection (Inglis *et al*., 2001); whereas parameters such as persistence, UV tolerance and conidial production have been overlooked. In the present study, the three best fungal isolates (lowest LC_{50} values) were compared for conidial production.

The *M. anisopliae* isolate ICIPE 69 produced significantly more conidia than the other isolates, which may be an advantage in terms of inoculum dispersion in the habitat, mass production and subsequent commercialization. The virulence of isolate ICIPE 69 against the legume flower thrips, *Megalurothrips sjostedti* Trybom (Ekesi *et al*., 1998), onion thrips, *Thrips tabaci* Lindeman (Maniania *et al*., 2003) and WFT (Maniania *et al*., 2002) has already

been demonstrated. These results coupled with high conidial production and tolerance to broad temperature range (Ekesi *et al*., 1999) makes it a suitable biopesticide candidate for thrips control.

The results of the ITS gene sequence amplification, showed two base pair differences in ICIPE 69 which corresponds the restriction site sequence *Serratia fonticola*. The nucleotide sequences of the three isolates suggest intra-specific genotypic variation (Mburu *et al*., 2011; Freed *et al*., 2011). Geographical origin of the fungal isolates may explain the variation observed in the present study. For instance, *M. anisopliae* isolate ICIPE 69 originated from the Democratic Republic of Congo whereas the other two originated from Kenya.

This restriction enzyme (SfoI) can be suggested as a tool for ICIPE 69 characterization using RFLP technique. A Basic Local Alignment Search Tool (BLAST) in NCBI, EMBL and Fungal Genome Search showed homology over 95% with *M. anisopliae*. However there is further need to characterize various fungal activity genes and their expression to relate the difference in activity to the molecular profile.

In conclusion, the efficacy of the *M. anisopliae* isolate ICIPE 69 on adult thrips and larval stages of WFT suggests its development as fungal biopesticide for thrips management.

CHAPTER FIVE

5.0 USE OF *METARHIZIUM ANISOPLIAE* CHITINASE GENES FOR GENOTYPING AND VIRULENCECHARACTERIZATION

5.1 Introduction

Entomopathogenic fungi are being exploited for insect pest control (Chandler et al., 2008; Ekesi and Maniania, 2007; Butt and Brownbridge, 1997). Among other advantages, entomopathogenic fungi infect by direct contact with insect cuticle without the need to be ingested like bacteria. To penetrate their hosts, several entomopathogenic fungi digest insect chitin by secreting chitinase proteins (St-Leger et al., 1996; Screen and St-Leger, 2000; Gao *et al*., 2011; Hu, 2005). These chitinases are also implicated in many aspects of fungal biology (Li, 2006; Hu, 2005; Musumeci and Paleotti, 2009; Arakane and Muthukrishnan, 2010; Rai and Kovics, 2010) including cellular processes such as conidial germination, hyphal growth and morphogenesis. Additionally chitinases contribute to defense against niche competitors and to nutrition by solubilising exogenous chitin fibers (Seidl *et al*., 2008; Seidl *et al*., 2009; Baker *et al*., 2009). Due to these critical functions, chitinase genes have been suggested as promising molecular markers for genotyping entomopathogenic fungi such as *Metarhizium anisopliae* (Enkerli *et al*., 2009; St-Leger *et al*., 1996; Bogo *et al*., 1998; Baratto *et al*., 2003; Kang *et al*., 1999).

The entomopathogenic fungus *M. anisopliae* produce different varieties of chitinases (Musumeci and Paleotti, 2009; Gimenez-Pecci *et al*., 2002; Baker *et al*., 2009; Barreto *et al*., 2004; Kang, *et al*., 1999). However, the role of each of these genes in the process of pathogenicity has been poorly established (Boldo *et al*., 2009). Many studies suggest that these multiple chitinases have a mutually synergistic and complementary effect in the process

of fungal infection. Recently, the characterized chitinase gene, *chi2*, cloned from strain E6 (Baratto *et al*., 2006) has been reported to be responsible for virulence in the genus *M. anisopliae* (Boldo *et al*., 2009). Over expression of *chi2* constructs showed higher efficiency in host killing, while the absence of the same chitinase diminished fungal infection efficiency (Boldo *et al*., 2009; Boldo *et al*., 2010).

In order to understand the genetic mechanisms underlying differences in virulence, eight ICIPE *M. anisopliae* isolates, which have shown difference in virulence on *F. occidentalis* and other insect pest were characterized using chitinase genes.

5.2 Materials and Methods

5.2.1 Fungus

Eight isolates of *Metarhizium anisopliae* ICIPE 7, ICIPE 20, ICIPE 30, ICIPE 41, ICIPE 62, ICIPE 63, ICIPE 69 and ICIPE 78 were selected for this study (Table 5. 1). These isolates have been previously bioassayed on 22 arthropod pests belonging to the orders of Diptera, Thysanoptera, Coleoptera, Isoptera and sub class Acari (Table 5.2). *Metarhizium anisopliae* var. *acridum* IMI330189, isolated from Niger and developed as a biopesticide for locust control, was included in the study as a reference.

Table 5. 1: List of *Metarhizium anisopliae* isolates investigated for their chitinase gene,

Species	Isolates	Locality (Country)	Source	Reference
M. anisopliae	ICIPE 7	Rusinga Island (Kenya)	*Amblyoma variegatum*	
	ICIPE 20	Migori(Kenya)	Soil	
	ICIPE 30	Kendu Bay (Kenya)	*Busseola fusca*	
	ICIPE 41	Migori (Kenya)	Soil	
	ICIPE 62	Matete (DRC)	Soil	
	ICIPE 63	Matete (DRC)	Soil	
	ICIPE 69	Matete (DRC)	Soil	
	ICIPE 78	Ungoye (Kenya)	*Temnoschoita nigroplagiata*	
	ARSEF 7524*	Switzerland		
	M34412*	India		
	E6*	Brazil		
M. anisopliae* var. *Acridum	IMI330189	Niger	*Ornithacris cavroisi*	
	ARSEF 324*	Australia		

***** ARSEF 7524, M34412, E6 and ARSEF 324 are out-groups used in BLAST for evaluating sequence similarity

5.2.2 DNA extraction, quantification and sequencing

Pure cultures of *M. anisopliae* isolates were produced on Sabouraud Dextrose Agar. Equal amounts (0.1 g) of conidia of each of the isolates were weighed in microcentrifuge tubes on a weighing balance (Mettler AT 261 Delta, Listers 2000). DNA was extracted from pure cultures of *M. anisopliae* isolates using a slight modification of the CTAB method (Doyle and Doyle, 1990). The *chi2* (chi2f/chi2r: GACAAGCACCCGGAGCGC/GCCTTGC TTGACACATTGGTAA) and *chi4* (chi4f/chi4r: GCTACTGGGAGAACTGGGAC/TTGTC GCCAARTGTCCARTT) gene fragments were amplified by PCR using published primers (Enkerli *et al*., 2009).

The chitinase genes chit1f/chit1r (chit1f/chit1r: CTCTGCAGGCCACTCTCGGT/AGCCATT GCTTCCTCATAT) and BbThchif and BbThhi (BbThchif/BbThchir: GGCTACTGGGAGA ACTGGGAC/TTGTCGCCAARTGTCCARTT) were also used in this study.

Each chitinase gene was amplified separately in 20 µl reactions comprising 1x PCR buffer (Genscript, Piscataway, NJ, USA), 2.5 mM of each dNTP (Genscript), 0.2 pm of each primer, $MgCl_2$ (2.5 mM), *Taq* DNA polymerase (0.5 units of Genscript) and genomic DNA (~25 ng). PCR amplification was performed in a PTC-100 thermocycler (MJR Inc., Minneapolis, MN, USA) using the following cycling parameters: 94 ℃ for 1 min, followed by 30 cycles of 94 ℃ for 1 min, 52 ℃ for 1 min, and 72 ℃ for 2 min, followed by a final elongation at 72 ℃ for 8 min. PCR products were separated by electrophoresis in a 1% ethidium bromide-stained agarose gel and visualized under ultraviolet light. The PCR products were purified using QuickClean DNA gel extraction kit (Genscript, Piscataway, NJ, USA) and sequenced at Macrogen (Korea).

5.2.3 Bioinformatics and phylogeny

Percent mortality and Lethal Time values data were filtered at a standard concentration and subjected to a k-mean clustering procedure (k = 4). Chitinase nucleotide sequences (*chi2* and *chi4*) were edited and aligned to remove ambiguous base calls before translating them to proteins using Geneious Software (Drummond *et al*., 2011). A search to identify protein sequences similar to *chi2* and *chi4* was performed using tBLASTx algorithm of NCBI GenBank. Geneious Software Amino acid sequences were used to estimate phylogeny with the neighbour-joining, minimum evolution or maximum parsimony methods. A dendrogram was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 with 10,000 bootstrap replicates. All methods gave trees with similar topology and approximate bootstrap values; therefore only the neighbor-joining tree is presented.

Percentage homology/genetic distance among similar chitinases to *chi2* and *chi4* were computed using MEGA software. The 3D structure was predicted using Swiss-PdB Viewer, v 4.0.1 (http://www.expasy.org/spdbv/). The conserved residues of the carbohydrate insertion domain (CID) (Li, 2010) were identified through multiple sequence alignment with the characterized chitinase genes.

5.3 Results

5.3.1 Clustering of *Metarhizium anisopliae* isolates using their virulence pattern and host range

ICIPE *M. anisopliae* isolates vary different in terms of virulence towards insect pests (Table 5. 2).

Table 5. 2 : Classification on *Metarhizium anisopliae* isolates based on their pathogenicity performance on various insect pests

	Target Pest		IMI330	ICIPE7	ICIPE20	ICIPE30	ICIPE41	ICIPE62	ICIPE63	ICIPE69	ICIPE78
Thysanoptera	Thrips	*F. occidentalis*		***	***	**	**			****	**
		Thrips tabaci								****	
		Megalurothrips								****	
Diptera	Leaf Miner	*L. huidobrensis*		***	****	**	**	***		***	*
	Fruit Fly	*C. rosa*	*		****		***	***	*	*	
		C. capitata	*		**		***	***		*	
		C. corsyra			**		**	**			
	Tse tse fly	*Glossina* sp				****					
	Mosquito	*Anopheles* sp				****					
	Sandfly	*P. duboscqi* sp									
Acari	Ticks	*Rhipicephalus* sp		****							
		Amblyoma sp									
	RSM	*T. evansi*		**	**	**	**	**	**	**	****
		T. urticae									****
Orthoptera	Locust	*Shistocerca* sp	****								
Isoptera	Termites	*Macrotermes* sp		***	**	***		***		**	
Coleoptera	weevils	***Cyclas sp.***									
Lepidoptera	Stemborers	***Chilo partellus***									
	LPB	***Maruka vitrata***									
Hemiptera	Corid bugs	***Helopelthis sp***									
Homoptera	Aphids	***Aphis* sp.**									

Pathogenic*, highly pathogenic**, virulent***, highly virulent****

In bold insect hosts that have been bioassayed with ICIPE *M. anisopliae*, but data were not considered in this study.

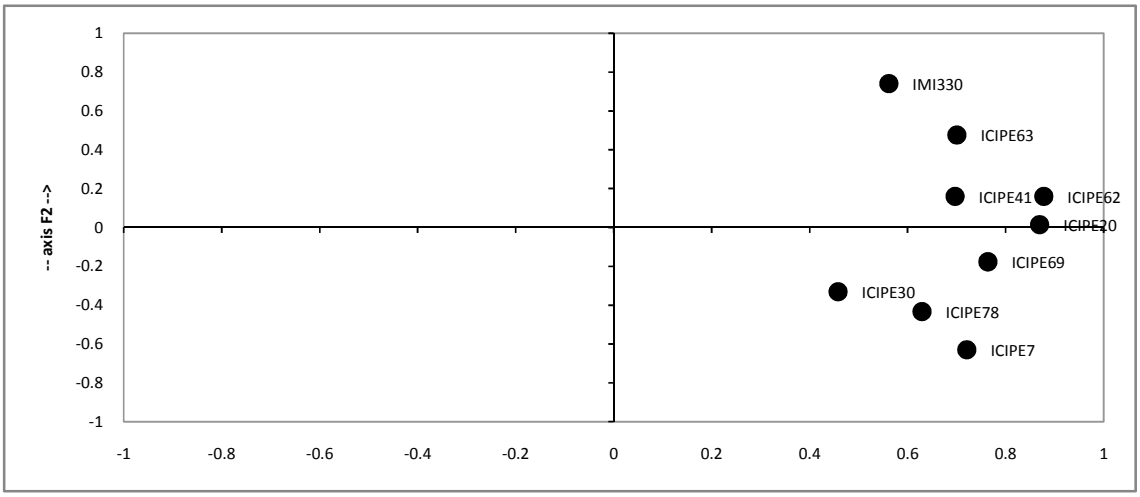

Figure 5. 1: Clustering of *Metarhizium anisopliae* isolates based on their virulence on 11 insect pests (k = 4).

The clustering of fungal isolates based on virulence data on 11 insect hosts showed different phenotypes (Figure 5. 1). The correlation matrix between *M. anisopliae* isolates showed that ICIPE 20 is correlated to IMI330189 and ICIPE 7, ICIPE 30 is correlated with ICIPE 7. There were correlations between ICIPE 41 and IMI330189 and between ICIPE 41 and ICIPE 20. ICIPE 62 is correlated with IMI330189, ICIPE 7, ICIPE 20 and ICIPE 41. ICIPE 63 is correlated with IMI330189, ICIPE 20 and ICIPE 41. ICIPE 69 is correlated with ICIPE 7, ICIPE 20, ICIPE 41, ICIPE 62 and ICIPE 63. ICIPE 78 is correlated with ICIPE 7, ICIPE 20, ICIPE 30, ICIPE 62 and ICIPE 69 (Table 5. 3).

At k = 4 (k-mean clustering), the clustering of *M. anisopliae* isolates based on insect-host showed different clusters. Cluster 1 includes fruit-fly species such as *Ceratitis rosa* Karsch and *Ceratitis capitata* Weidemann. In cluster 2 ornamental pest are represented e. g. *Frankliniella occidentalis*, *Megalurothrips sjostedti* Trybom, *Liryomiza huidobrensis* Blanchard and *Tetranichus urticae* Koch. In cluster 3 comprises five hosts: *Ceratitis cosyra* Walker, *Phlebotomus duboscqi* Neveu-Lemaire, *Tetranichus evansi* Baker and Pritchard,

Macrotermes michaelensi Sjostedt and *Cylas puncticollis* Boheman. Cluster 4 had the biggest number of hosts (11) containing all insects described in three first clusters (Table 5. 4).

Table 5. 3 : Correlation between *Metarhzium anisopliae* based on virulence (% Mortality and LT values) on 11 insect pests

	IMI330	ICIPE7	ICIPE20	ICIPE30	ICIPE41	ICIPE62	ICIPE63	ICIPE69	ICIPE78
IMI330		-0.062	**0.499**	0.012	**0.509**	**0.611**	**0.746**	0.298	0.033
ICIPE7			**0.617**	**0.538**	0.401	**0.532**	0.205	**0.662**	**0.726**
ICIPE20				0.393	**0.608**	**0.765**	**0.616**	**0.661**	**0.541**
ICIPE30					0.266	0.349	0.163	0.408	**0.431**
ICIPE41						**0.637**	**0.564**	**0.504**	0.369
ICIPE62							**0.691**	**0.642**	**0.483**
ICIPE63								**0.451**	0.234
ICIPE69									**0.557**
ICIPE78									

In bold, significant values at the level of significance alpha=0.050 (two-tailed test)

Table 5. 4 : Composition of the *Metarhizium anisopliae* clusters

Clusters	**Cluster1**	**Cluster2**	**Cluster3**	**Cluster4**
Within-groups inertia	**0.01**	**8.27**	**9.63**	**73.18**
Size	**2**	**4**	**5**	**11**
	C. rosa *C. capitata*	*F. occidentalis* *Megalurothrips* *L. huidobrensis* *T. urticae*	*C. cosyra* *P. duboscqi* *T. evansi* *Macrotermes* *Cyclas*	*F. occidentalis* *Megalurothrips* *L. huidobrensis* *C. rosa* *C. capitata* *C. cosyra* *P. duboscqi* *T. urticae* *T. evansi* *Macrotermes* *Cyclas*

5.3.2 Sequence alignment

All *M. anisopliae* had the four chitinase genes (Plate 5.1). Comparison of the *chi2* nucleotide sequences from all selected *M. anisopliae* isolates from different parts of Africa showed that there was no difference in the open reading frames (of eight isolates) composed of 229 amino acid residues. However, when compared with similar chitinase sequences retrieved from NCBI database, differences in amino-acid composition were identified (Figure 5. 2).

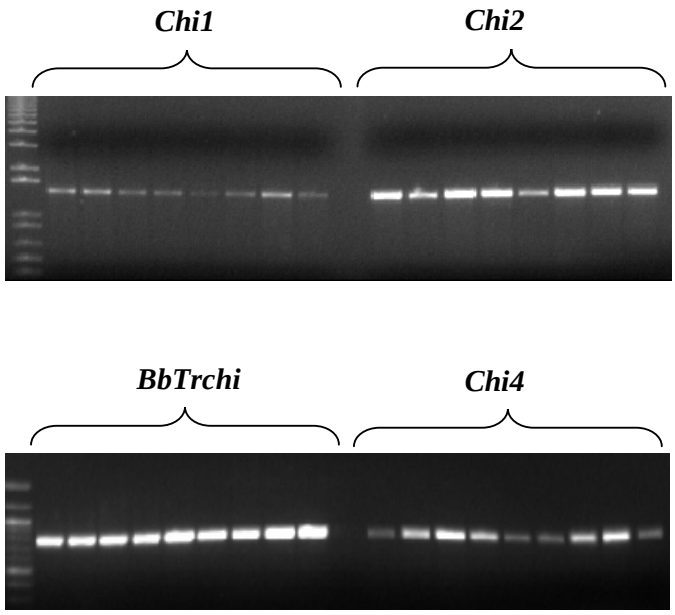

Plate 5. 1: PCR products electrophoresed through 1% EtBr-stained agarose gel of *chi1* (1.4kb), *chi2* (1.3kb), *BbTrchi* (0.9 kb) and *chi 4* (0.8 kb) from left to right: ladder 1kb fermentas, IMI330189, ICIPE 7, ICIPE 20, ICIPE 30, ICIPE 41, ICIPE 62, ICIPE 63, ICIPE 69 and ICIPE 78

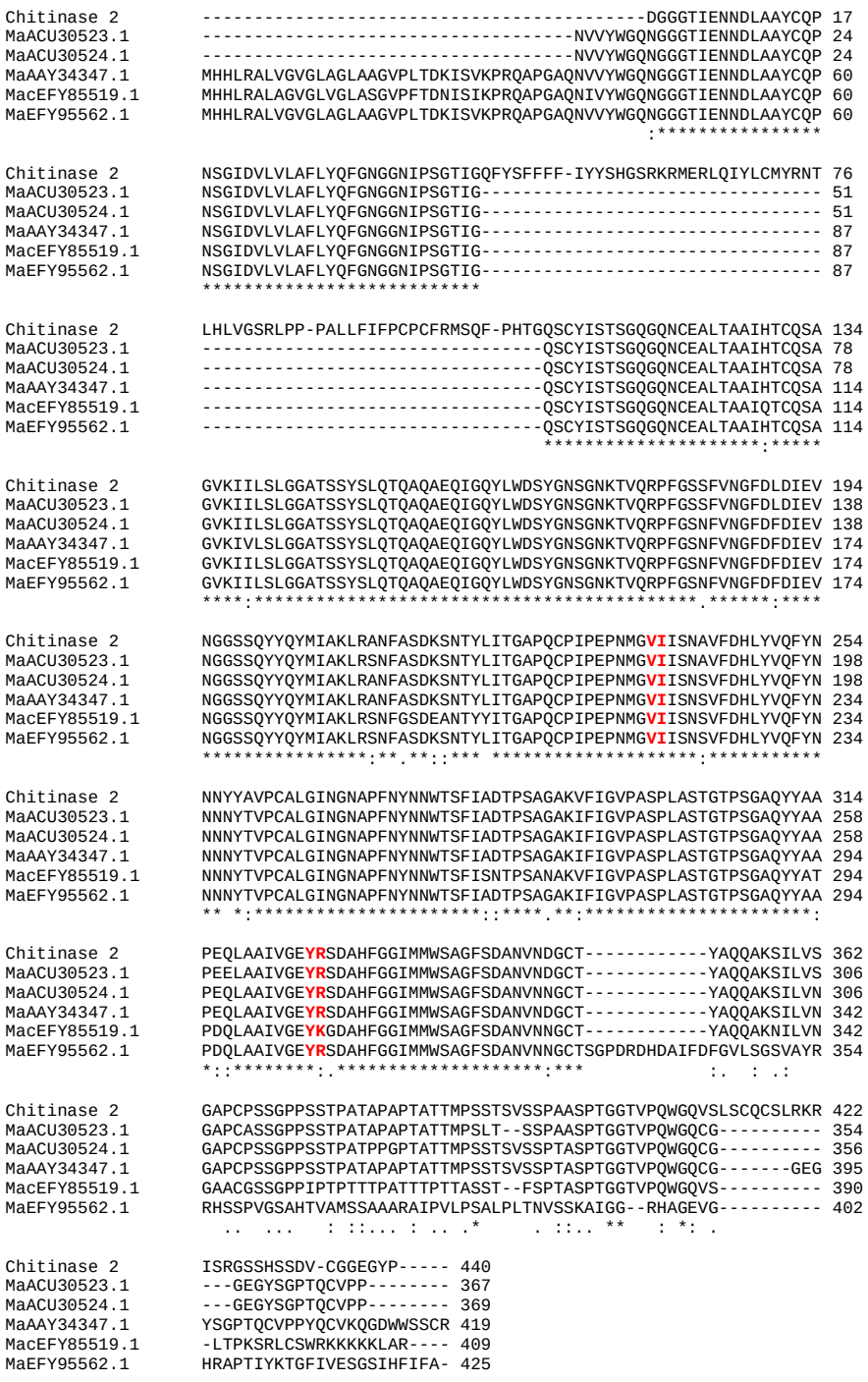

Figure 5. 2: The multiple sequence alignment (Clustal W v2.1) showing relationship between the Chitinase 2 with similar sequences obtained from the NCBI. The initials represent the species (Ma; *Metarhizium anisopliae,* Mac; *Metarhizium anisopliae* var. *acridium*) followed by their accession numbers as provided in the GenBank. Highlighted residues in red (VI and YR) show the conserved residues of the CID.

The phylogenic analysis shows over 100% identity in *chi2* sequences. *Metarhizium anisopliae* var. *acridum* EFY85519.1 was different from the four outgroups of *M. anisopliae* including the *chi 2* template from the tested isolates and the three out-groups ACU30524.1; AAY34347.1 and ACU30523.1. *Metarhizium anisopliae* EFY95562.1 were completely different from the rest of the *M. anisopliae* isolates (Figure 5.3).

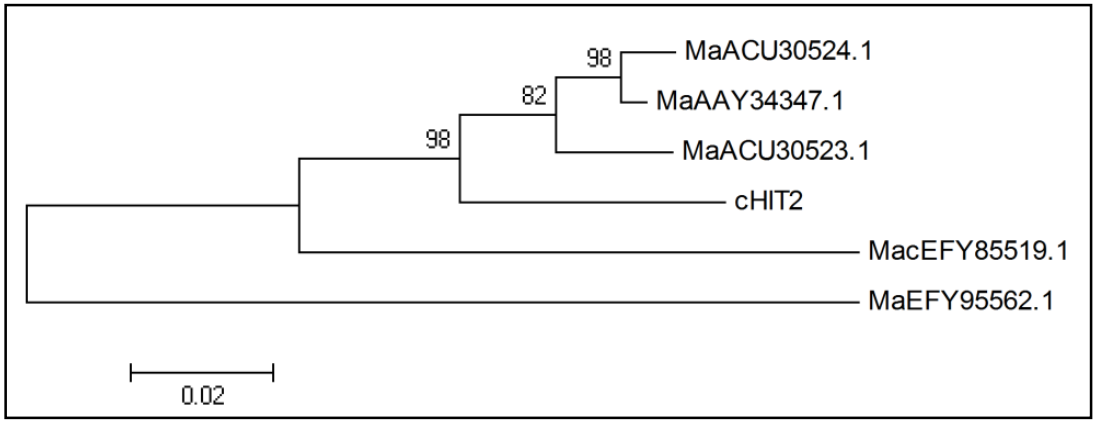

Figure 5. 3 : A dendrogram showing relationships between the *chi 2* gene and the related sequences retrieved from the NCBI GenBank.

5.3.3 Homology modelling of chitinase2

The Swiss-Pdb Viewer (http://www.expasy.org/spdbv/) server was used to predict the 3D structure of *chi2*. The conserved residues of the CID (VI and YR) was present (Figure 5. 4) in all eight *M.* var. *anisopliae* isolates that exhibited no differences in their coding regions. However, in *M. anisopliae* var. *acridum* the 'YR' motif is replaced by 'YK'.

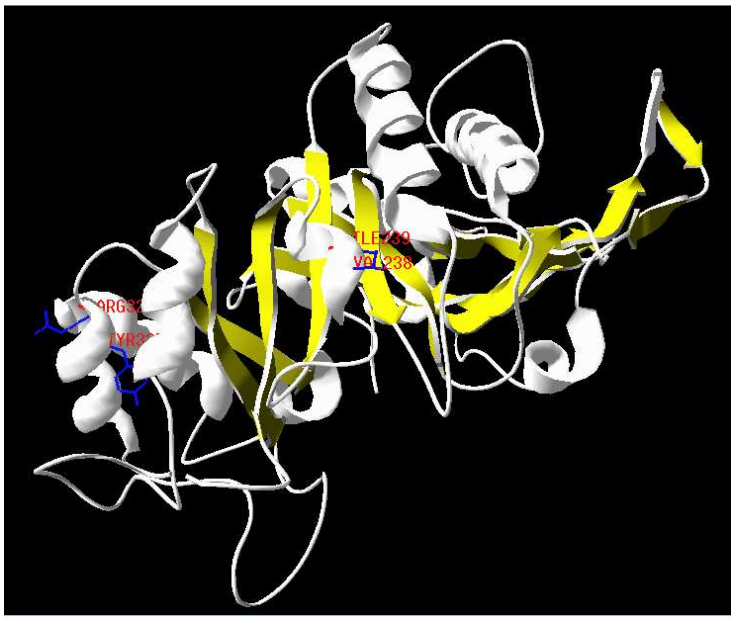

Figure 5. 4 : Chitinase 2 model as predicted using the Swiss-PdB Viewer. The residues highlighted (Val238 and Ile239; Tyr325 and Arg326) represent conserved residues in the Carbohydrate Insertion Domain (CID) of chitinases.

5.3.4 Analysis of Chitinase genes *chi 4*

All eight *M. anisopliae* var. *anisopliae* isolates had identical chitinase 4 nucleotide sequence. After the editing process to remove the ambiguous base calls a BLAST analysis using *chi4* sequence on NCBI GenBank database, revealed highest amino acid identities to *M. anisopliae* var. *anisopliae* M34412, ARSEF7524 and *M. anisopliae* var. *acridum* IMI330189 (Figure 5. 5).

Figure 5. 5 : Multiple sequence alignment (ClustalW, v2.1) showing the relationship between the Chitinase 4 with similar sequences obtained from the NCBI. The initials represent the species followed by their accession numbers as represented in the GenBank.

5.4 Discussion

The ICIPE *M. anisopliae* strains isolated from different regions of Africa had different levels of virulence on various insect pests. The clustering analyses based on virulence data describe various categories of phenotypes in terms of virulence among isolates. ICIPE 20 is known to be highly pathogenic on insect pests like fruit flies and leaf miners (Dimbi *et al*., 2003; Migiro *et al*., 2010), while ICIPE 69 has been identified as the most promising isolate in the control of thrips (Ekesi and Maniania, 2000a; Ekesi *et al*., 1998; Dimbi *et al*., 2003; Maniania *et al*, 2002; Ngakou *et al*., 2008). Cluster 3, on the other hand, includes isolates which are more pathogenic to flies, termites, and mites. ICIPE 7, ICIPE 30 and ICIPE 62 isolates fit into cluster 3 because they are highly pathogenic on the two-spotted spider mites;

Tetranychus urticae and *Macrotermes michaelseni* (Mburu *et al*., 2009; Bugeme *et al*., 2009; Mburu *et al*., 2011). ICIPE 30 was used successfully to control tsetse fly *Glossina* spp. in the field, (Maniania, 1998; Maniania *et al*., 2002) and was also reported to be virulent on the banana weevil, *Cylas puncticollis* (Ondiaka *et al*., 2008). ICIPE 7 showed high pathogenicity on mites *Tetranychus urticae Tetranychus evansi* and ticks, *Rhipicephalus appendiculatus* and *Rhipicephalus pulchellus* (Nana *et al*., 2010; Nchu *et al*., 2009). Furthermore, it was reported to be a potential isolate for the control of larval stages of *F. occidentalis* (Niassy *et al*., Unpublished). Lastly, cluster 4 groups a category of virulence demonstrating that all the above investigated ICIPE isolates can be pathogenic to some extend on wide group of pest.

Enkerli *et al*. (2009) suggested the use of chitinase genes as a new tool that would be useful for genetic characterization of *M. anisopliae* strains. In the present study, the comparison of chitinase sequences, *chi2* and *chi4*, among the various *M. anisopliae* isolates did not show differences in nucleotide sequence that could be exploited for genotyping. *M. anisopliae* var. *acridum* IMI330189, that was included in the study for comparison purposes, had slight sequence differences with the ICIPE isolates at the *chi4* locus. We were however unable to amplify the *chi2* geno of this isolate.

The predicted protein 3D structure of the *chi2* shows similar folding patterns to the characterized chitinases and possesses the conserved CID domain of most chitinases. Since the *chit2* gene sequence was identical in all the 8 studied ICIPE isolates, only one 3D model is presented (Figure 5. 4). The analysis of the CID conserved residue showed exactly the same *chi2* structure describing probably the same functionality (Li, 2010; Li, 2006; Moreira *et al*., 2010).

Enkerli *et al*., (2009) used PCR-RFLP technique for genotyping *M. anisopliae* var. *anisopliae* strains. Our results did not corroborate with their findings, which reported that chitinase genes could discriminate *M. anisopliae* strains. Moreover, those findings were not related to the pathogenic potentiality of *M. anisopliae* strains.

All ICIPE *M. anisopliae* isolates that were used in this experiment showed the same *chi2* and *chi4* gene sequences, despite the fact that they originated from different countries in Africa. Only IMI330189 (*M. anisopliae* var. *acridum*), which originated from Niger, showed a nucleotide substitution in the *chi4* sequence, that was non-synonymous.

Chitinase gene *chi2* has been reported to be mainly responsible for *M. anisopliae* virulence (Boldo *et al*., 2009). This would imply that all the investigated isolates with same *chi2* gene should have the same virulence pattern to different insect pests. In light of results, two hypotheses arise: chitinase genes are either differentially regulated (i.e. different expression levels) in different isolates or there are other parameters that affect the process of infection. Regarding the first hypothesis, *chi2* gene has been reported to be up-regulated by chitin (which serves as a carbon source to the fungus) and in conditions of fungus autolysis, and is down-regulated by glucose (Baratto *et al*., 2006). Chitin composition of insect cuticle can affect chitinase production level (Gołębiowskia *et al*., 2007 #268) which would justify the difference in virulence. Since insect pests have special cuticle composition, virulence may vary accordingly. Moritz *et al*. (1997) reported that adult thrips and larvae to have different cuticle structures, which could explain, in part, the difference in susceptibility to entomopathogenic fungi (Ugine *et al*., 2005; Maniania *et al*., 2002; Vestergaard, 1995). On the other hand, the *chi2* gene contains 70 bp introns, consequently, Boldo *et al*. (2010) reported a possibility of splicing of the *chi2* protein suggesting that there are different

transcripts that are generated from the same DNA sequence. This information does not explain the variability observed on pathogenicity among different isolates reported here. This study suggests the presence of other relevant factors that affect fungal virulence. For exemple, factors such as conidiation genes, toxin production genes are often overlooked. Niassy *et al*. (unpublished data) observed that ICIPE 69 produces more conidia than ICIPE 20 and ICIPE 7 which were also virulent to on *Frankliniella occidentalis* larvae. Fang *et al*. (2007) demonstrated that gene disruption of conidiation-associated gene (cag8) in *M. anisopliae* results in the lack of conidia on agar plates and on infected insects, reduced mycelial growth, decreased virulence suggesting the involvement of cag8 is involved in the modulation of conidiation, virulence and hydrophobin synthesis in *M. anisopliae*.

Metarhizium anisopliae strains are being screened and selected using classic bioassays for their virulence for biopesticide development. Although chitin digestion is a critical step in the success of fungal infection, the use of chitinase genes for genotyping might not be appropriate for virulence characterisation. Other parameters need to be taken into consideration for the characterisation of fungal virulence. This study suggests further work including chitinase gene expression profiling and *in vitro* chitin digestion procedures to compare quality and quantity of chitinase production between various ICIPE isolates.

CHAPTER SIX

6.0 COMPATIBILITY OF *METARHIZIUM ANISOPLIAE* ISOLATE ICIPE 69 WITH AGROCHEMICALS USED IN FRENCH BEAN PRODUCTION

6.1 Introduction

French bean, *Phaseolus vulgaris* L. (Fabaceae), is one of the most important vegetables exported from East Africa. In Kenya, it accounts for over 60% of all export crops (Nderitu *et al*., 2007). French bean is a host to a wide range of insect pests including aphids, coleopterans and thrips (Nuez and Prohens, 2007). The Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande), is the most important thrips in East Africa causing considerable damage to production of French bean in Kenya. Losses of 40-60% at the farm and 20% at the collecting points have been reported (Nderitu *et al*., 2007). In addition to insect pests, French bean is also vulnerable to many diseases caused by bacteria, fungi and viruses (Nuez and Prohens, 2007). Presently, the commonly resorted option to control these insects and diseases is to apply synthetic chemical insecticides, acaricides, fungicides, and to some extent botanical insecticides. However, WFT is believed to have developed resistance to all the major classes of chemical insecticides (Jensen, 2004).

In recent years, entomopathogenic fungi have been developed as microbial insecticides as alternatives to synthetic chemical insecticides for the control of many insect pests including thrips (Butt and Goettel, 2000; Chandler *et al*., 2008). *Metarhizium anisopliae* (Metschnikoff) Sorokin isolate ICIPE 69 is among the fungal pathogens under development for the control of WFT and other of thrips species including *Megalurothrips sjostedti* Trybom and *Thrips tabaci* Lindeman in East Africa (Ekesi *et al*., 1998; Ekesi and Maniania, 2000; Maniania *et al*., 2002; Maniania *et al*., 2003). Since French bean is host to various arthropod pests and fungal diseases, which require application of synthetic pesticides to control them, it is

necessary to evaluate the compatibility of entomopathogenic fungi based biopesticides with these commonly used synthetic pesticides in the context of integrated pest management (Ekesi and Maniania, 2000; Irigaray *et al*., 2003; Da Silva and Neves, 2005). In the present study, we investigate the effects of 12 agrochemicals commonly used in French bean production on *M. anisopliae* isolate ICIPE 69 in terms of vegetative growth, conidia production, mycelial mass and virulence against second-instar larvae of *F. occidentalis*.

6.2 Materials and Methods

6.2.1 Agrochemicals

Twelve agrochemicals commonly used in French bean production were selected to assess their compatibility with *M. anisopliae* isolate ICIPE 69. They included five insecticides, two acaricides, three fungicides and one botanical pesticide (Table 6. 1).

6.2.2 Vegetative growth

Hundred (100) µl of conidial suspension titrated at 10^6conidia ml^{-1} was spread-plated on Potato Dextrose Agar (PDA) medium and incubated at 25 °C. After 48h post-inoculation, a plug (0.4-cm diameter) was removed using a cock borer and placed on PDA plates previously mixed v/v of each chemical. Orthogonal lines were drawn on plates to monitor the growth of the mycelial plug. Data for vegetative growth were recorded at 5, 11 and 19 days after insertion of the plugs. No chemical was added in the control treatments, and all treatments were repeated six times. Plates showing contamination were discarded.

Table 6. 1: List of main agrochemicals used in French bean pest management

Chemicals	Trade Name	Active Ingredient	Formulation	Target pests	Group
Insecticides	Actara – Syngenta	Thiamethoxam	250g/kg WDG	Citrus thrips	Neonicotinoid
	Duduthrine (Karate)	L-cyhalothrin	EC 1.75g/l	Lepidoptera, Hemiptera, Diptera, Coleoptera	Pyrethroid
	Confidor - Bayer Crop	Imidacloprid	SC200	Aphid, mirids, brown flea, whitefly sucking insect	Neonicotinoid
	Dursban - Dow AgroSciences	Chlorpyrifos	EC 480 g/l	Ants, Aphids, Armyworms, mites, fleas, aphids, ticks	Organophosphate
	Murphy - Murphy Chemicals	Diazinon	EC30-60ml/20l	Horticultural pest, fruit trees pest	Organophosphate
Botanical insecticides	Achook	Azadirachtin	EC 0.15%	Nematicide/insecticide	Limonoid
	Neemrock	Azadirachtin	EC 0.03%	Vegetable pest (Diamondback moth)	Limonoid
Acaricides	Dynamec - Syngenta	Abamectin	EC 1.8	Two spotted spider mites, Thrips, Psylla	Naturally derived insecticide/acaricide
	Oberon - Bayer Crop	Spiromesifen	SC 240ga.i./l	White fly, spider mite and psyllids	Keto-enol
Fungicides	Goldazim - Collin Campbell	Carbendazim	SC 500	fungi	Benzimidazole carbamate
	Kocide – Dupont	Copper Hydroxide		Bacteria and Fungi	Copper fungicide
	Milraz WP76 - Dow AgroSciences	Probineb + Cymoxamil	700g/kg 60g/kg	fungi	Dithiocarbamate + ethyl urea

EC=Emulsifiable concentrate, SC= Suspension Concentrate, WDG=Water Dispersible Granule.

6.2.3 Mycelial mass

Petri dishes used for vegetative growth were kept up to three weeks after insertion of the plug and the mycelial mat was harvested using a spatula and immediately weighted and then placed in an oven at 50°C for 30 minutes to assess the dry weight.

6.2.4 Conidia production.

Potato Dextrose Agar plates previously mixed v/v of each chemical were inoculated with 48h-old *M. anisopliae* plugs as described earlier. The mycelial mat was harvested three weeks after inoculation and suspended in 10ml 0.05% Triton then vortexed for 1 mn to obtain homogeneous suspension. Conidia were quantified using Neubauer counting chamber.

6.2.5 Effects of combining imidacloprid and thiamethoxam with *Metarhizium anisopliae* ICIPE 69 on the susceptibility to second-instar larva of *Frankliniella occidentalis.*

Three concentrations of imidacloprid and thiamethoxam (10%, 20%, and 50% of the recommended concentrations) were combined with three concentrations of *M. anisopliae* (10^6, 10^7 and 10^8 conidia ml^{-1}). Recommended doses (100%) of imidacloprid (0.5ml/l) and thiamethoxam (0.2g/l) were included as a check. French bean pods were surface-sterilized in 3% sodium hypochlorite and rinsed thrice in sterile distilled water before being used. Treatments consisted of soaking French bean pods in various suspensions for 10 seconds. Pods were then transferred to paper towel and allowed to dry for 5-10 minutes. Treated pods were later transferred individually in 10-ml glass tubes containing paper towel to allow insect pupation and absorb the excess moisture from the pod. Twenty second-instar WFT larvae were introduced in the tube containing treated French bean. Test-insects were maintained at

25 °C and 70% humidity for 8 days. The tube was closed using a lid with a hole of 1-cm diameter covered by a thrips proof mesh to allow ventilation. Mortality was recorded daily for eight days and the experiment was repeated four times.

6.2.6 Data analysis

Data were arcsine transformed for normalization and analyzed using SAS, (SAS Institute 2002-2003). Compatibility (T) was calculated according to Alves's formula: T= [((20 × VG) + (80 × S))/ 100] (Alves et al., 1998); whereby, the values for the vegetative growth and the spore production (S) are given in percentage in relation to the control; where T from 0 to 30 = very toxic; 31 to 45 = toxic; 46 to 60 = moderately toxic; 60 to 90 = compatible; > 90 = highly compatible. A correlation between the vegetative growth, the conidia production, the mycelial mass and the mycelial dry mass was performed and a Principal Component Analysis (PCA) was used to confirm the effect of chemicals on the fungus. Percentage mortality (at 7 days post-treatment) was also adjusted for natural mortality in controls using Abbott (1925) formula before analysis and was then analyzed using ANOVA (SNK). The LT_{50} values were determined for each replicate using the probit analysis method and compared among themselves using ANOVA followed by mean separation using Student-Newman-Keuls (SNK) test, (P = 0.05).

6.3 Results

6.3.1 Vegetative growth

At recommended dose, the vegetative growth of *M. anisopliae* varied significantly between the treatments at 5 day ($F_{12, 282}$ = 97, P < 0.001), 11 day ($F_{12, 277}$ = 195, P < 0.001), and 19 day

($F_{12, 283}$ = 252; P < 0.001) post-inoculation. With exception to 19 days post-inoculation where the vegetative growth in the control and imidacloprid was similar, the vegetative growth in the control was significantly higher than in the other treatments (Table 6.2). No vegetative growth was recorded with carbendazim.

6.3.2 Conidia production

The conidia production varied significantly among the treatments ($F_{12, 277}$ = 19.1; P < 0.0001). There was no significant difference between the control, abamectin, imidacloprid and thiamethoxam. There was significant difference between the control and the other treatments. Carbendazim caused the most deleterious effect and was significantly different from the rest of the treatments (Table 6. 3).

6.3.3 Mycelial mass

The mycelial mass was higher in the control and L-Cyhalothrin treatments than in the other treatments ($F_{12, 277}$ = 109.0, P < 0.0001) (Table 6. 3; Plate). *Metarhizium anisopliae* in association with L-Cyhalothrin produced the highest dry weight (284.4 mg), followed by the control (147.2 mg) (Table 6. 3).

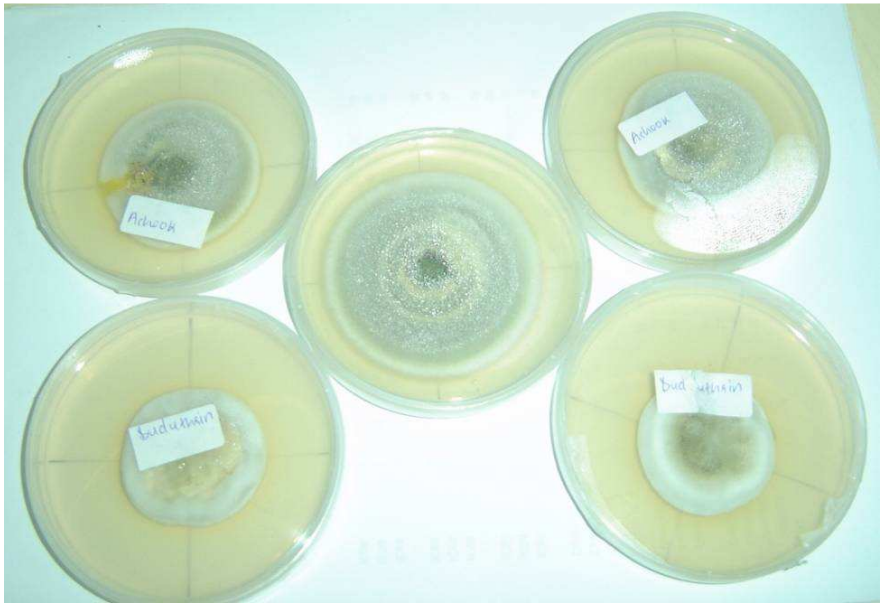

Plate 6. 1 : Effect of Achook, Duduthrine on radial growth of *Metarhizium anisopliae* ICIPE 69

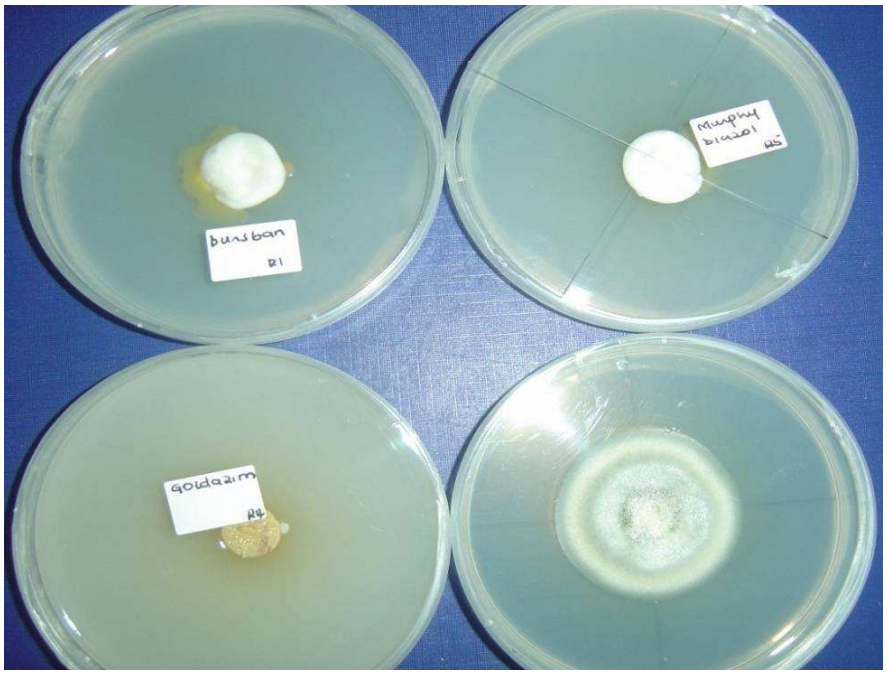

Plate 6. 2 : Effect of Dursban, Goldazim Murphy and Neemrock on *Metarhizium anisopliae* radial growth and spore production.

Table 6. 2: Effect of 12 selected agrochemicals on vegetative growth of *Metarhizium anisopliae* ICIPE 69 at 5, 11 and 19 days after inoculation at 25 °C

Chemicals	Vegetative growth (mm)		
	Days after inoculation		
	5	**11**	**19**
Control	8.6 ± 2.3a	17.4 ± 0.3a	29.2 ± 0.3a
Abamectin	6.7 ± 0.2b	12.5 ± 0.3cd	22.7 ± 0.9c
Azadirachtin 0.03%	5.6 ± 0.2c	11.2 ± 0.7ed	18.9 ± 1.1d
Azadirachtin 15%	6.8 ± 0.2b	13.7 ± 0.2bc	21.4 ± 0.3c
Carbendazim	0.0 ± 0.0f	0.0 ± 0.0h	0.0 ± 0.0i
Chloropyrifos	2.3 ± 0.2e	5.1 ± 0.3gf	11.2 ± 0.3g
Copper Hydroxide	2.8 ± 0.2e	5.8 ± 0.4f	11.1 ± 0.4g
Diazinon	2.3 ± 0.2e	4.3 ± 0.3g	9.2 ± 0.5h
Imidacloprid	6.6 ± 0.3b	14.3 ± 0.4b	29.3 ± 0.4a
L-Cyhalothrin	5.7 ± 0.3b	10.8 ± 0.3e	16.5 ± 0.3e
Probineb	3.8 ± 0.3d	12.4 ± 0.3cd	13.1 ± 0.5f
Spiromesifen	5.4 ± 0.2c	12.4 ± 0.3cd	21.6 ± 0.8c
Thiamethoxam	5.3 ± 0.2c	12.7 ± 0.4cd	25.7 ± 0.5b
	F = 97, DF= 12, 282; P < 0.001	F = 195, DF = 12, 277; P < 0.001	F = 252, DF = 12, 283; P < 0.001

Means in columns followed by the same letters are not significantly different by SNK

Table 6. 3: Conidia production and mycelial mass, of ICIPE 69 exposed to recommended doses of 12 agrochemicals on SDA for three weeks after plug insertion

Chemicals	Conidial production × 10^8	Mycelial mass	M. Dry mass
Control	14.0 ± 0.8a	338.2 ± 16.9a	147.2 ± 10.7b
Abamectin	14.3 ± 1.3a	90.8 ± 0.9cd	34.0 ± 1.9c
Azadirachtin 0.03%	4.7 ± 0.8b	142.3 ± 4.5b	64.0 ± 7.3c
Azadirachtin 0.15%	2.9 ± 0.8b	100.7 ± 13.0cd	34.1 ± 3.1c
Carbendazim	0.0 ±0.0c	0.0 ± 0.0f	0.0 ± 0.0c
Chloropyrifos	0.6 ± 0.1b	74.4 ± 8.0ed	28.6 ± 4.0c
Copper hydroxide	1.2 ± 0.1b	70.8 ± 4.0ed	54.7 ± 4.6c
Diazinon	0.1 ± 0.0b	98.2 ± 7.7cd	28.6 ± 4.0c
Imidacloprid	14.1 ± 3.9a	118.0 ± 52.0bc	54.6 ± 2.9c
L-Cyhalothrin	3.6 ± 0.3b	318.0 ± 13.0a	284.4 ± 75.0a
Propineb	1.2 ± 0.1b	52.4 ± 6.0e	46.1 ± 16.1c
Spiromesifen	5.4 ± 1.1b	73.3 ± 2.0ed	34.4 ± 1.4c
Thiamethoxam	9.9 ± 1.0a	125.0 ± 4.0bc	47.0 ± 0.5c
	F=19.1; DF=12, 282; P<0.0001	F=109.0; DF=12, 277; P<0.0001	F=10.7; DF=12, 283; P<0.0001

Means in columns followed by the same letters are not significantly different by SNK

6.3.4 Classification of toxicity of agrochemicals against *Metarhizium anisopliae* isolate ICIPE 69 according to Alves model

According to the Alves's model, the *M. anisopliae* ICIPE 69 was highly compatible with abamectin, imidacloprid and compatible with thiamethoxam. Spiromesifen showed a moderately toxic effect, while azadirachtin and L-cyhalothrin were toxic to the fungus (Table 6. 4). Chlorpyrifos, carbendazim, diazinon, copper hydroxide and probineb were very toxic to the fungus. There was a strong correlation between vegetative growth and conidia production (Pearson, $r = 0.9$; $P = 0.0002$), and between the mycelia mass and the mycelia dry mass (Pearson, $r = 0.9$; $P < 0.0001$); whereas there was no strong correlation between the mycelial mass and conidial production (Pearson, $r = 0.4$; $P = 0.15$) (Table 6. 5). The principal component analysis (PCA) showed that imidacloprid, abamectin and thiamethoxam can be grouped as chemicals with no effects on vegetative growth and conidia production of the *M. anisopliae* (Figure 6.1). On the other hand, azadirachtin and the fungicides, copper hydroxide and propineb, affected the vegetative growth and conidia production of the *M. anisopliae*. The fungicide carbendazim had the most deleterious effect among all the agrochemicals tested. Spiromesifen which was moderately toxic in Alves model centred between the compatible and the toxic chemicals (Figure 6.1).

Table 6.4: Compatibility of 12 selected agrochemicals with *Metarhizium anisopliae* ICIPE 69, according to Alves model, (Alves et al., 1998).

Chemicals	VG	SP	T	Classification
Abamectin	77.5	102.3	97.4	Highly Compatible
Azadirachtin 0.03%	64.7	33.6	39.9	Toxic
Azadirachtin 15%	73.2	21.1	31.5	Toxic
Carbendazim	0	0	0	Very Toxic
Chloropyrifos	38.1	4.2	11.0	Very Toxic
Copper Hydroxide	38.0	8.5	14.4	Very Toxic
Diazinon	31.5	1.0	7.1	Very Toxic
Imidacloprid	100.2	101.2	101.0	Highly Compatible
L-Cyhalothrin	56.5	25.5	31.7	Toxic
Probineb	44.9	8.5	15.8	Very Toxic
Spiromesifen	73.8	38.7	45.7	Moderately Toxic
Thiamethoxam	87.8	70.9	74.2	Compatible

VG= Vegetative growth; SP= Spore production; T= Compatibility value

Table 6. 5: A Pearson correlation (r; p) between 4 parameters: vegetative growth, conidia production, mycelial mass and dry mass.

	Mycelia mass	Mycelia Dry mass	Conidia production
Vegetative growth	0.5; 0.07	0.3; 0.4	0.9; 0.0002
Mycelia mass		0.9; <0.0001	0.4; 0.15
Mycelia dry mass			0.2; 0.6

P > |r| under H0: Rho=0

6.3.5 Effects of imidacloprid and thiamethoxam on the virulence of *Metarhizium anisopliae* ICIPE 69 to second-instar larvae of *Frankliniella occidentalis*.

Mortality caused by imidacloprid alone varied between 44.3% (10% of recommended concentration) and 94% (recommended concentration) and the one by thiamethoxam ranged between 33% (10% recommended concentration) and 92% (recommended concentration) (Table 6. 6). *Metarhizium anisopliae* applied alone caused mortalities of 34, 54 and 76% at the concentrations of 10^6, 10^7 and 10^8 conidia ml^{-1}, respectively (Table 6. 6). At 10^6 conidia ml^{-1}, the combination with imidacloprid and thiamethoxam at 10, 20 and 50% was different from the fungus alone in term of mortality. Although the combination of different concentrations of *M. anisopliae* with different concentrations of the two chemicals did not affect the virulence of the fungus, it did not result in any synergism (Table 6. 6). However, the combination of thiamethoxam at the concentration of 10% with *M. anisopliae* at the concentration of 10^6 conidia ml^{-1} resulted in short LT_{50} of 6 days as compared to 13 days with thiamethoxam alone and 11 days with the fungus alone (Table 6. 7).

Table 6. 6: Mean Mortality (± SE) of second instar larvae of *Frankliniella occidentalis* treated with *Metarhizium anisopliae* ICIPE 69 in combination with different rates of imidacloprid and thiamethoxam.

Chemicals		Conidial concentrations ml^{-1}			
		0	**10^6**	**10^7**	**10^8**
Imidacloprid	0% RC	-	33.5 ± 4.6b	54.0 ± 2.1a	76.2 ± 2.1a
	10%RC	44.3 ± 10.9b	69.6 ± 11.3a	66.5 ± 11.9a	77.0 ± 11.7a
	20%RC	74.0 ± 14.5ab	74.8 ±11.3a	78.5 ± 8.6a	82.2 ± 6.4a
	50%RC	86.3 ±10.8a	75.0 ±1.8a	88.5 ± 9.6a	89.0 ± 4.8a
	100%RC	94.0 ± 3.5a	-	-	-
		F=4.94, P=0.03, DF=3	F=5.63, P=0.01, DF=3	F=2.86, P=0.08, DF=3	F=0.68, P=0.58, DF=3
Thiamethoxam	10%RC	33.3 ±9.2c	59.2 ± 8.5a	65.0 ±9.0a	82.2 ± 10.5a
	20%RC	55.7 ± 13.9bc	64.5 ± 6.6a	74.2 ± 5.9a	91.0 ± 6.0a
	50%RC	73.0 ± 6.8ab	76.0 ±10.5a	75.2 ± 4.2a	91.8 ± 4.8a
	100%RC	92.2 ± 6.0a	-	-	-
		F=8.17; P=0.006, DF=3	F=5.18, P=0.016, DF=3	F=2.86, P=0.08, DF=3	F=1.28, P=0.32, DF=3

RC: Recommended Dose of the chemical on French bean crop per litre of water imidacloprid and thiamethoxam respectively 0.5ml/l and 0.2g/l.

Within a column means followed by the same letters are not significantly different Student-Newman-Keuls.

Table 6. 7: Median Lethal Time LT50 (days) of *Metarhizium anisopliae* ICIPE 69 formulated with different rates of imidacloprid and thiamethoxam on second instars larvae of *Frankliniella occidentalis*

Chemicals	Conidial concentrations				
	Rates	0	10^6	10^7	10^8
Imidacloprid	0% RC	-	11.0 ± 0.1aA	6.5 ± 0.4aB	4.0 ± 0.6aB
	10% RC	8.4 ± 1.0aA	5.0 ± 0.5bA	5.9 ± 1.0bA	5.0 ± 1.1aA
	20% RC	4.3 ± 0.7bA	4.8 ± 1.0bA	3.7 ± 0.6bA	3.8 ± 0.2aA
	50% RC	3.7 ± 0.3bA	4.0 ± 0.1bA	3.3 ± 0.2bA	3.3 ± 0.3aA
	100% RC	2.3 ± 0.1b	-	-	-
Thiamethoxam	10%RC	12.5 ± 5.4aA	6.2 ± 1bAB	5.8 ± 0.9abAB	3.5 ± 0.5aB
	20%RC	6.4 ± 1.4aA	5.1 ± 0.2bA	4.3 ± 0.6bA	3.4 ± 0.5aA
	50%RC	4.4 ± 0.2aA	4.8 ± 0.9bA	4.3 ± 0.2bA	3.1 ± 0.4aA
	100%RC	2.6 ± 0.4a	-	-	-

RC = Recommended concentration.

Means within the same row with the same capital letters are not significantly different by SNK

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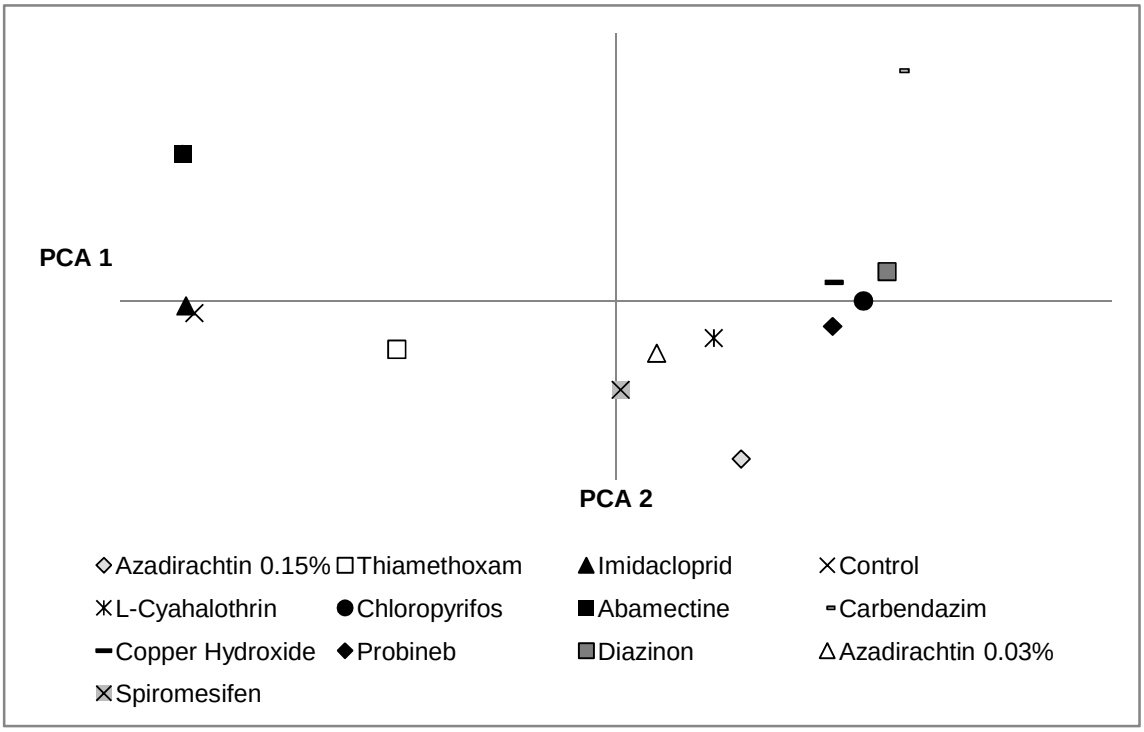


Figure 6.1: Principal Component Analysis: Effects of 12 agrochemicals on the vegetative growth, spore production, mycelial mass of *Metarhizium anisopliae* ICIPE 69 on PDA at 25 °C.

6.4 Discussion

A wide range of agrochemicals including botanical and synthetic chemical pesticides are applied to control pests and diseases in French bean. The agrochemicals commonly used to control arthropod pests and diseases in French bean showed various effects on the entomopathogenic fungus *M. anisopliae* isolate ICIPE 69. For instance, azadirachtin, the fungicides, L-cyhalothrin and chlorpyrifos were toxic to the fungus (Plate 6.1). Chlorpyrifos has already been reported to negatively affect *M. anisopliae*, probably due the presence of chlorus (Li and Holdom, 1995). On the other hand, combination of chlorpyrifos with sublethal concentrations of *M. anisopliae* has been reported to have additive synergistic

effects on German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae) (Pachamuthu and Kamble, 2000). Both synergistic and antagonistic effects of azadiracthtin have been reported against several isolates of *Beauveria bassiana* (Balsamo), (Mohan *et al*., 2007). Depieri *et al*., (2005) and Rachappa *et al*., (2007) reported the inhibition of entomopathogenic fungi by azadirachtin. Although high mycelial mass and dry mass was produced with L-Cyhalothrin, it was classified as toxic to the *M. anisopliae* ICIPE 69. The negative effect of L-Cyhalothrin on entomopathogenic fungi has been reported earlier (Olajire and Oluyemisi, 2009). The deleterious effects of carbendazim on *M. anisopliae* mycelia growth and conidia production observed in this study are similar to the findings of Moorhouse *et al*., (1992) and Rachappa *et al*., (2007) on the same fungus (Plate 6.2). The acaricides showed varied effects on the *M. anisopliae* ICIPE 69 as reported earlier by Shi et *al*., (2005). For instance, abamectin was highly compatible while spiromesifen moderately toxic. Abamectin has also been reported to be compatible to entomopathogenic fungi (Tamai *et al*., 2002). Imidacloprid and thiamethoxam did not have deleterious effects on the vegetative growth and conidia production. Compatibility of the two neonicotinoids with entomopathogenic fungi has already been reported by many workers (Filho *et al*., 2001; Neves *et al*., 2001; Wenzel *et al*., 2004). Although the combination of these two chemicals with the fungus did not affect the virulence of the fungus, no synergism was observed, except with the association of the fungus at the concentration of 10^6 conidia ml^{-1} and thiamethoxam at the concentration of 10%, resulting in short LT_{50}. Dara and Hountondji (2001) also reported the lack of synergism between imidacloprid and *Hirsutella thompsonii* Fisher against the cassava green mite *Mononychellus tanajoa* Bondar. However, many studies have shown that imidacloprid significantly increases insect pest susceptibility when combined with *M. anisopliae* (Ramakrishnan *et al*., 1999; Ansari *et al*., 2007; Santos *et al*., 2007).

This study shows that some of the synthetic chemical pesticides and botanical insecticides used to control insects and diseases in French bean have negative effects on the *M. anisopliae* ICIPE 69. Therefore, it will not be advisable to apply them at the same time with *M. anisopliae* ICIPE 69 meant for the control of WFT. On the other hand, imidacloprid and thiamethoxam can be suggested in French bean production in combination with the fungus. Field studies to evaluate the compatibility of these agrochemicals with the fungal isolate applied either as combinations, or incorporated singly with the isolate will bring additional information on how *M. anisopliae* ICIPE 69 can be successfully incorporated in IPM systems together with the insecticides.

CHAPTER SEVEN

7.0 EFFECTS OF HOST-PLANT ON *METARHIZIUM ANISOPLIAE* INFECTION TO *FRANKLINIELLA OCCIDENTALIS* PERGANDE (THYSANOPTERA: THRIPIDAE)

7.1 Introduction

The Western Flower Thrips (WFT), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), is an economically important pest in the world. It is polyphagous and can thrive on more than 250 plant species belonging to over 60 families, including French bean, Cowpea, Snow pea (Lewis, 1997b). In Kenya, yield losses by WFT can often reach 60% of the harvest due to direct feeding and yield loss, rejection at farm gate and quarantine restrictions issues (Nderitu *et al.*, 2008). Synthetic chemical insecticide sprays have always been the principal method of thrips management (Lewis, 1997a). Other approaches using cultural practices and plant resistant varieties are also being recommended to farmers (Nderitu et al., 2007). However, the use of chemical pesticides is no longer sustainable due to Maximum Residue Limit imposed by the European Union and environment hazards. Moreover, thrips have developed resistance to many synthetic chemical insecticides (Jensen, 2004). Entomopathogenic fungi are among the most promising alternative to chemical pesticides being developed for the control of thrips (Ekesi and Maniania, 2000; Butt and Brownbridge, 1997). *Metarhizium anisopliae* ICIPE 69 is already under registration for the control of thrips in ornamental system.

A number of French bean varieties with different commercial value and levels of tolerance to thrips damages are cultivated in Kenya (Nderitu *et al*., 2007). Plants produce a diverse array of chemicals some with defensive properties (Koschier, 2006; Teulon *et al*., 2010; Ugine *et*

al., 2007). Furthermore, the host plant on which the insect thrive on may confer insect resistance to fungal infection through the process of sequestration (Ugine *et al*., 2007).

The variability of released compounds between varieties could influence fungal efficacy (Ekesi *et al*., 2000).

The role of tritrophic interactions plant host, insect host and entomopathogen in biological control has often been overlooked. Therefore, the aim of this study was to determine the effects of intra and interspecific host-plant variations on the virulence of *M. anisopliae* to western flower thrips.

7.2 Material and methods

7.2.1 Plants

Three (3) French bean cultivars, *Samantha*, *Alexandria* and *Julia* and snow pea were used in this study. Crops were planted in plastic pots in field-cages (3 m length by 3 m width by 2.2 m height), using a mixture of manure and soil in a ratio of 1-5, respectively. Crops were grown in the greenhouse and bean pods and flowers were used as food supply for thrips.

Plate 7. 1: Pods of host-plants and experimental conditions

7.2.2 *Frankliniella occidentalis* colony

The rearing methods were details widely in section 3.2. *Frankliniella occidentalis* were reared up to three generations on the four host plants. Second instar- larvae were used in the experiments.

7.2.3 *Metarhizium anisopliae*

Metarhizium anisopliae isolate ICIPE 69 was used in the present study. It was described in Chapter 4.

7.2.4 Effect of host-plant on the virulence of *Metarhizium anisopliae on Frankliniella occidentalis*

Ten (10) ml of concentrations of 1×10^6; 1×10^7; 1×10^8 conidia ml^{-1} was sprayed on four pods of the four host-plants using a Burgejon spray tower (Burgejon, 1956). Tests insects were handled as described in previous sections. Each treatment was randomized and each treatment was repeated four times.

7.2.5 Data analysis

Percent mortality was corrected for control mortality (Abbott, 1925 #1) and normalized by arcsine-transformation before being subjected to analysis of variance (ANOVA) using PROC GLM, at 95% level of significance. Student-Newman-Keuls analysis was used to separate the means as a post-ANOVA procedure. Median lethal time (LT_{50}) and median lethal concentration (LC_{50}) were estimated using a Probit model. These analyses were carried out using GENMOD procedure of SAS version 9.2. Fiducial limits overlappings (95% confidence intervals) were used to identify differences among the values of LT_{50}.

7.3 Results

There were significant differences in mortality between the four host- plants ($F = 4.6$; $P = 0.008$). There were a significant differences in mortality between the *M. anisopliae* concentrations ($F = 23.6$; $P = 0.0001$). However there was no significant interaction between the two factors host-plants and the *M. anisopliae* doses ($F = 0.8$; $P = 0.6$).

7.3.1 Effect of host-plant on the virulence of *Metarhizium anisopliae* on second instar larvae of *Frankliniella occidentalis*

At low concentration 1×10^6 conidia ml^{-1} of *M. anisopliae*, there was no significant difference between the French bean varieties. However there were no significant differences between snow pea and the three French bean varieties.

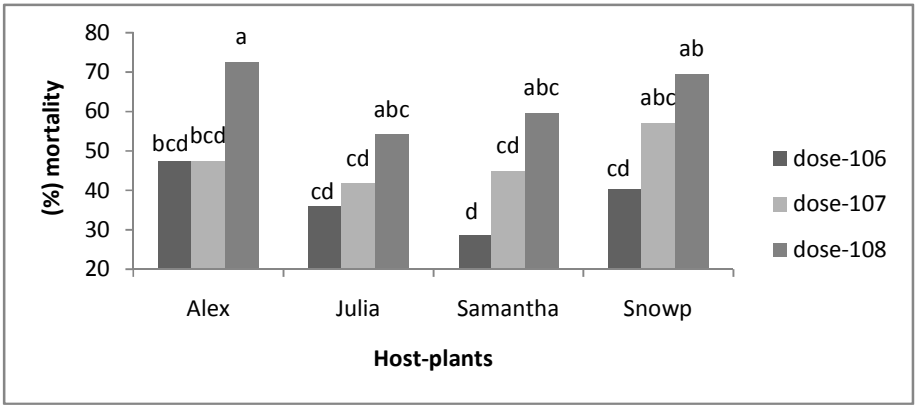
Table 7. 1: Factorial ANOVA describing the effect of Host plants on the virulence of *Metarhizium anisopliae* on *Frankliniella occidentalis*

Treatments	DF	Fisher's F	Pr > F
Host plants	3	4.6	0.008
M. anisopliae concentrations	2	23.6	< 0.0001
Host plant**M. anisopliae*	6	0.8	0.6

However, the interaction between host plants and the *M. anisopliae* concentrations was not significant (F = 0.8; P = 0.6).

At 1×10^7 conidia ml^{-1} of *M. anisopliae*, there was no significant difference between the 3 varieties of French bean and snow pea.

At high concentration 1×10^8 conidia ml^{-1} of *M. anisopliae*, there was no significant difference between the French bean varieties and snow pea (Figure 7. 1).



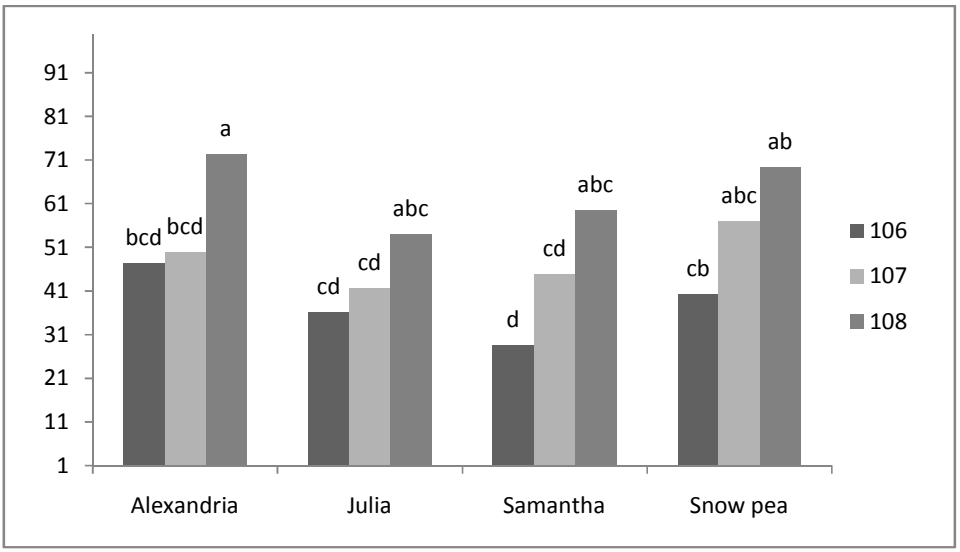


Figure 7. 1 : Host plant effect on *Metarhizium anisopliae* virulence on *Frankliniella occidentalis*. With the same fungal treatment, means bearing same letters are not significantly different in SNK.

The analysis of the LT_{50} values showed that at 1×10^6 conidia ml^{-1} *Alexandria* and *Julia* had significantly shorter LT_{50} values as compared to *Samantha*. The LT_{50} value for snow pea was not significantly different from *Alexandria* and *Julia*, but significantly shorter than *Samantha* (Table 7.2).

At 1×10^7 conidia ml^{-1} *Alexandria* had the shortest LT_{50} as compared to *Julia* while it was not significantly different from *Samantha* and Snow pea.

At 1×10^8 conidia ml^{-1} *Alexandria* had the shortest LT_{50} and was significantly different with the other host-plants. There were also significant difference between *Julia* and *Samantha*. There was no overlapping between *Alexandria* and *Samantha*. There were no significant difference between snow pea and *Samantha*. However snow pea was significantly different with *Julia* and *Alexandria* (Table 7. 2).

Table 7. 2: Median Lethal time of *M. anisopliae* applied on *Frankliniella occidentalis* reared on different host-plants

Host-Plants	*M. anisopliae* concentrations (conidia ml^{-1})					
	1×10^6		1×10^7		1×10^8	
	LT_{50}	Fid. Limit	LT_{50}	Fid. limit	LT_{50}	Fid. limit
Alexandria	8.6	8.1 – 9.1	7.8	7.4 – 8.2	5.7	5.4 – 5.9
Julia	9.2	8.7 – 10.0	9.0	8.5 – 9.6	7.1	6.9 – 7.5
Samantha	11.7	10.7 – 13.2	8.4	8.0 – 9.0	6.7	6.4 – 7.0
Snowpea	9.6	9.1 – 10.1	7.6	7.3 – 8.0	6.9	6.6 – 7.0

Table 7. 3: Overall median Lethal time (LT_{50} in days) of 2nd instar larvae of *Frankliniella occidentalis* treated with 3 different doses of *Metarhizium anisopliae*; 1×10^6; 1×10^7; 1×10^8 conidia ml^{-1}.

Host-plants	LT_{50} Days	(95%) Fiducial limits	Probit model
Alexandria	7.2	7.0 – 7.4	*P = Probit (– 1.70 + 0.23 × Days)*
Julia	8.3	8.0 – 8.6	*P = Probit (– 1.96 + 0.23 × Days)*
Samantha	8.7	8.4 – 9.0	*P = Probit (– 1.87 + 0.21 × Days)*
Snow pea	7.9	7.8 – 8.1	*P = Probit (– 2.09 + 0.26 × Days)*

Median lethal time (LT_{50}) with overlapping fiducial limits is not significantly different

The overall comparison of the LT_{50} values between the host-plants showed that *Alexandria* had the shortest LT_{50} value (7.2) and was significantly different with *Julia* and *Samantha* respectively 8.3 and 8.7. There was no significant difference between *Samantha* and *Julia.* Compared to snow pea there were no differences in LT_{50} between snow pea and *Julia.* However there were significant difference between *Alexandria* and Snow pea and between Snow pea and *Samantha* (95% confidence interval) (Table 7. 3).

7.4 Discussion

The effect host-plant on fungal virulence on insect pest in often overlooked. In terms of tolerance to *F. occidentalis* a study carried out by Nderitu *et al.* (2008) showed that the pod damage score was similar between *Julia* and *Samantha* and the mean total number of thrips was approximately the same which would implies that insect susceptibility to fungal treatment when reared on those two varieties would be approximately the same. This was not the case in our study; although there were no significant differences in mortality (Figure 7.1), *F. occidentalis* was more susceptible when reared on *Julia* than on *Samantha* (Table 7. 2). The variability of released compounds among plant varieties can influence fungal efficacy. For instance, Ekesi *et al.* (2000) reported that some effects of airborne volatiles and crude extracts of tolerant variety revealed an inhibitory effect on fungal germination, colony forming units and growth.

The overall comparison of LT_{50} values showed that thrips raised with the French bean varieties *Alexandria* were more susceptible than those fed with *Julia* or *Samantha*. The French bean variety *Alexandria* is a newly introduced tolerant variety in Kenya for export. It has been previously reported that food quality can increase insect resistance to fungal infection for instance which could partly explain thrips susceptibility. The difference of pest susceptibility when reared on different species of host-plant has been reported by many authors (Migiro *et al*., 2011).

Moreover, parameters such as differential levels of substrates humidity or rates of conidial acquisition (pick-up) from treated plant surfaces have been reported to affect tremendously fungal virulence (Poprawski *et al*., 2000). For instance, WFT reared on snow pea crop seems to be as susceptible as those raised on French bean varieties *Alexandria* and *Julia*. In this

study the selected host-plants varied in terms of shape size and shelf-life. These variations can result in subsequently differences in susceptibility to *M. anisopliae* infection in terms of conidia acquisition and food quality. For instance, it has been shown that thrips fed on plant pollen are less susceptible than thrips fed on leaves (Ugine *et al*., 2007).

This study demonstrated that *M. anisopliae* virulence can be affected by inter and intra specific variations of host-plants. Results suggest that *M. anisopliae* application rate should be adjusted during the control *F. occidentalis* in French bean varieties such as *Samantha* and *Julia* as compared to French bean var. *Alexandria*, and Snow pea. This variability of susceptibility of *F. occidentalis* to different host-plants should be taken into consideration during pest management.

CHAPTER EIGHT

8.0 PERFORMANCE OF SEMIOCHEMICAL-BAITED AUTOINOCULATION DEVICE WITH *METARHIZIUM ANISOPLIAE* THE CONTROL OF *FRANKLINIELLA OCCIDENTALIS* ON FRENCH BEAN IN FIELD-CAGE

8.1 Introduction

Frankliniella occidentalis (Pergande) is a pest of many cultivated crops, causing high economic losses. It is also a vector of tospovirus diseases on many cultivated crops (Lewis, 1997b). Due to their minute size and cryptic habit, thrips are very difficult to manage. Synthetic chemical pesticides are the most common strategy used for the control of thrips. The ongoing use of these chemical pesticides is however unsustainable due to their adverse effects on the environment and the development of resistance among thrips species (Jensen 2004). This coupled with the stringent measures such as the Maximum Residue Limits set up by the European Union on export produce has led to the search for more environmental-friendly alternatives.

Entomopathogenic fungi are among the alternatives being considered. One isolate of *Metarhizium anisopliae* (Metchnikoff) Sorokin ICIPE 69 which has been reported to be highly pathogenic on major thrips pests (Maniania *et al*., 2002; Ekesi and Maniania, 2000a; Ngakou *et al*., 2008) is in the process of registration in Kenya for the control of *F. occidentalis.* Inundative release is so far the only mode of application of the insect pathogen for the control thrips in a cropping system (Jaronski, 2010).

Semiochemicals; pheromones and allelochemicals: allomones; kairomones and synomones, initially developed as tools for thrips monitoring are now being investigated for thrips control *occidentalis,* are attracted to semiochemicals (Teulon *et al*., 2008;

Koschier, 2006; Koschier, 2008). In greenhouse conditions, 18 – 20 folds increase in trap captures of thrips have been reported (van Tol, *et al*., 2007). In the open field situation when tested on French bean crop this attractant was found to enhance the trap captures from 2 – 6 folds depending on the prevailing climatic conditions (Muvea *et al*., 2010). Since thrips, including *F. occidentalis*, are attracted to semiochemicals in very high numbers (Teulon *et al*., 2008; Koschier, 2006; Koschier, 2008), it may be possible to apply the autodissemination strategy, whereby insects that are attracted to a semiochemical-baited inoculation device are infected with the pathogen before they return to the environment where they can disseminate the pathogen among host populations (Dowd and Vega, 1995). Such an approach has already been developed against fruit flies (Quesada-Moraga *et al*., 2008; Dimbi *et al*., 2003); tsetse flies (Maniania, 2002; Maniania *et al*., 2006; Maniania, 1998) and recently against leaf miner (Migiro *et al*., 2010). The aim of this study was therefore to assess the performance of a semiochemical-baited inoculation device of *anisoplaie* for possible control of *F. occidentalis* on French bean in the screenhouse.

8.2 Materials and methods

8.2.1 *Frankliniella occidentalis* colony

Insect colony has been details in section 3.2

8.2.2 Field-cages and French bean crops

French bean *Phaseolus vulgaris* var. *Samantha* was used in this experiment. The crops was planted in plastic pots in 50-µm mesh field-cages (3 m length by 3 m width by 2.2 m height) (Amiran LDT, Kenya), using a mixture of compost and cow-dung manure and soil in a ratio

of 1-5, respectively. Experiments were conducted between July and August 2010. Fifty pots were used per field-cage and the plants were used at flowering stage 45 days after planting. A “Tiny Tag” data logger (Gemini (2003-2007 UK) Ltd) was placed in the middle of the field cage to measure diurnal temperature and humidity during the trial. The device was set for four reading every 6 hours a day.

8.2.3 Fungal isolate

Metarhizium anisopliae isolate ICIPE 69 was described in Chapter 4.

8.2.4 Thrips kairomone

A commercial semiochemical for thrips, Lurem-TR, was obtained from Pherobank, Wagenigen, Netherlands. The active substance is an ethyl-isonicotinate a pyridine compound and has previously been reported to be an effective monitoring tool for *F. occidentalis* (Davidson *et al*., 2007).

8.2.5 Autoinoculation device for *Metarhizium anisopliae*

The autoinoculation device has been described in chapter 3.3.4. In addition, a 9.3-cm blue plastic disc was added on the top and bottom of the device to increase the attraction (Chen *et al*., 2004). The semiochemical dispenser was inserted inside the smaller plastic diameter bottle (see Migiro *et al*., 2010).

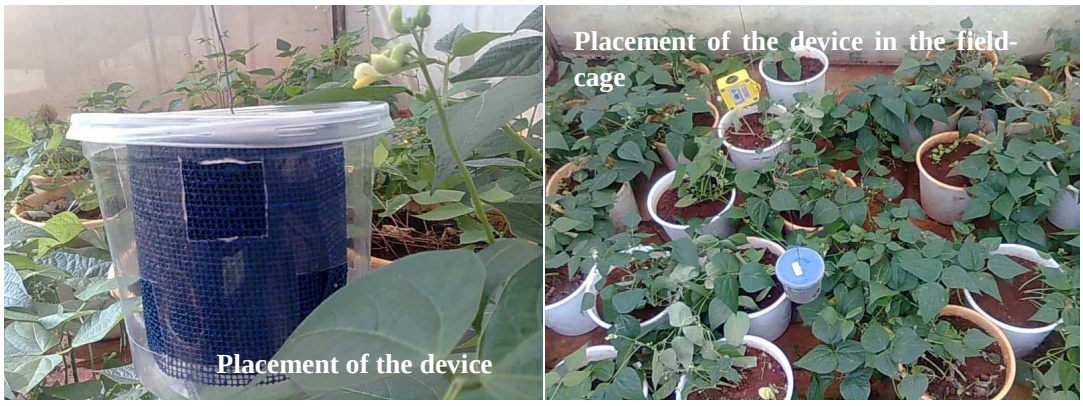


Plate 8. 1: Autoinoculation device and device setting in a French bean crop at flowering stage in Field cage

Approximately 3 g of dry conidia was spread evenly on the velvet cloth of the autoinoculation device. The blue netting was then wrapped around the velvet cloth containing spores and tightened with two office pins. The device was then hanged at canopy level (35 cm) at the middle of the field-cage (9 m^2, 2.2 m height). Five thousand (5000) adult thrips were released in each field cage containing French bean plants at flowering stage. Treatments were randomized and the experiment was replicated four times over time.

8.2.6 Infectivity of *Metarhizium anisopliae* in autoinoculation semiochemical-baited device

The treatments consisted of (i) fungus-treated device with semiochemical, (ii) fungus-treated device without semiochemical, and (iii) fungus-free device as control. At 1, 2, 3, 4, 5, 6 and 7 days post-exposure, 50 adult thrips were randomly collected from each of the treatment field cages by tapping a whole plant using a barber-tray and an aspirator. Twenty (20) live thrips were transferred separately using a cotton-plugged aspirator into 10-ml clean sterile glass tubes (2 cm×10) each containing a fresh bean pod as food and brought to the laboratory.

Tubes containing thrips were maintained in an incubator at 25 ± 2°C and 80-90% RH. Mortality was recorded daily for 7 days. Dead insects were placed in humidified chamber to allow for the development of mycosis on the surface of cadaver. The remaining 30 thrips were used to estimate the conidial acquisition which is number of conidia picked up by a single thrips in each treatment. Insects were transferred individually into 2-ml cryogenic tubes containing 1ml of sterile 0.05 % Triton X-100. The tube was then vortexed for 2-3 min to dislodge conidia from the insect and concentration of conidia determined using a haemocytometer. Thrips density per plant was also assessed at 7 days post-inoculation. Ten plants were randomly selected for each treatment and the whole plant was tapped five times and all thrips falling off the plant were collected using a barber tray and an aspirator. Insects were brought to the laboratory where they were counted. The experiment was replicated four times over time.

Plate 8. 2: Thrips sampling technique using a barber tray and aspirator

Plate 8. 3: Thrips conidial acquisition and mortality assessment in laboratory

8.2.7 Conidial persistence

A moist cotton bud was used to collect samples of conidia daily (1-7 post-inoculation) from the auto-inoculation devices in the two treatments. The end of the cotton bud containing conidia was cut and suspended in a 10-ml 0.05% Triton X-100 and vortexed for 1 min. One hundred (100) µl of the solution was spread-plated on SDA plates and incubated for 16 hours in incubator at 25 ± 2 ^{o}C. Percentage germination of conidia was determined by counting the number of germinated conidia (a germ tube two times the diameter of the propagule) from 100 spores counted randomly on the surface area covered by each cover slip under the light microscope according to Goettel and Inglis (1997). Four replicate plates per treatment were used.

8.2.8 Statistical analysis.

Thrips mortality data were corrected for control mortality (Abbott, 1925) and arcsine normalized before being subjected to analysis of variance (ANOVA) using PROC GLM, at 95% level of significance and Student-Newman-Keuls (SNK) analysis was used to separate the means. A Pearson correlation was used to determine correlation between

thrips mortality and other parameter such as conidial persistence. Temperature and humidity data were collected using the Tinytag Explorer software. A linear regression model was used to study the interactions between mortality and related factors (conidial persistence, conidial acquisition, temperature and humidity).

8.2.3 Results

The overall mean number of conidia acquired per single thrips sampled from thrips taken from field cages after 7 days post-inoculation was significantly higher in semiochemical-baited device ($5.0 \pm 0.6 \times 10^4$ conidia/insect) than in the device without semiochemical ($2.2 \pm 0.4 \times 10^4$ conidia/insect) (ANOVA: $F_{1,42} = 15.9$, $P = 0.0002$).

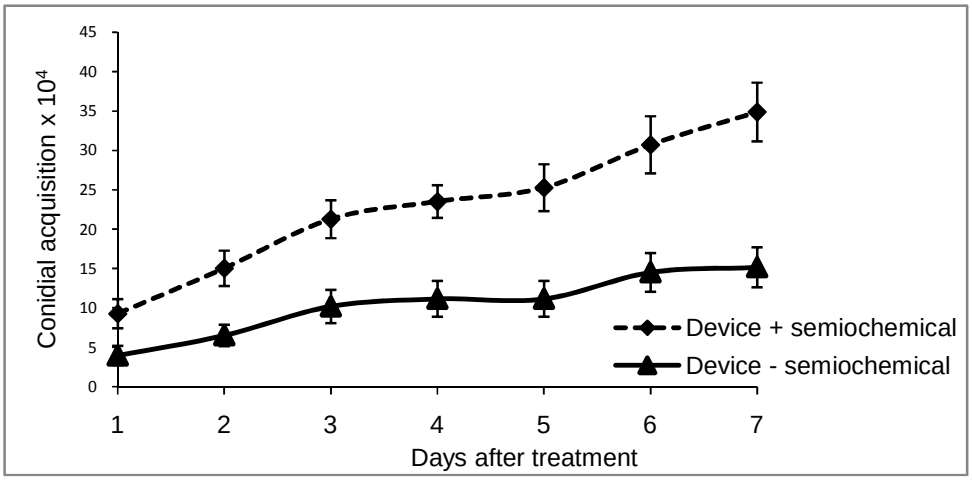


Figure 8. 1: Cumulative mean number of conidia picked by single thrips in field-cages at 1 to 7days after placement of *Metarhizium anisopliae* treated devices with and without semiochemical.

The cumulative mean number of conidia picked by single thrips over time is presented in Figure 8. 1. Control mortalities in all the treatments varied between 5.7 and 7.0 % (95%

confidence interval). The overall mean percentage mortality of thrips taken from field cages 7 days post-inoculation was 59.3 ± 3.9% in *M. anisopliae* treated semiochemical-baited device and 41.7 ± 3.5% in *M. anisopliae* treated free semiochemical-baited device, which was significantly different (ANOVA: $F_{1,42} = 10.9$, P = 0.0017).

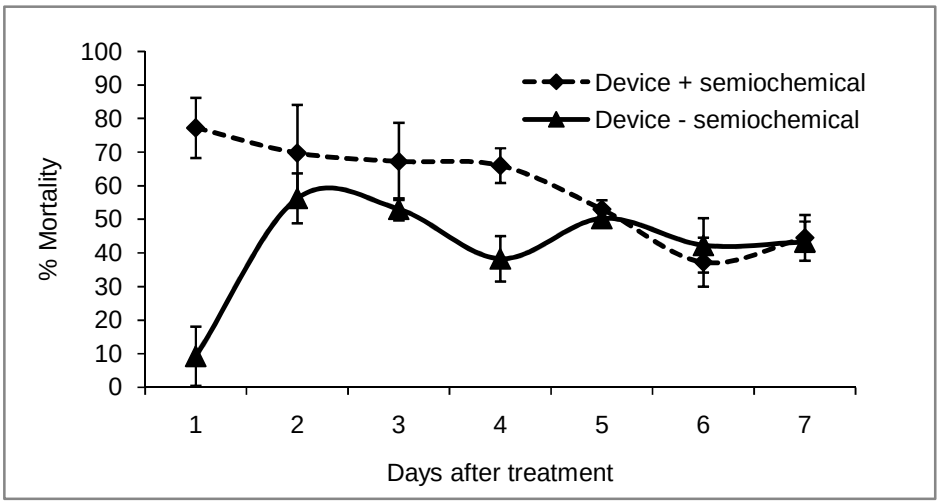


Figure 8. 2: Mortality (%) *Frankliniella occidentalis* adults collected from field cages at 1 to 7days after placement of an auto-inoculation device in the field cage.

At day 1 post-treatment, mortality of thrips taken from field cages in *M. anisopliae* free semiochemical-baited device treatment was significantly low (9.2%) compared to *M. anisopliae* treated semiochemical-baited device (77.2%) (Paired t-test: t = 2.4, P = 0.002) (Figure 8. 2). At day 2 and day 3 post-treatment, there were no significant differences in mortality between *M. anisopliae* treated semiochemical-baited device and *M. anisopliae* free semiochemical-baited device treatment respectively 69.8 and 56.2.0% (Paired t-test: t = 2.4, P = 0.4), 67.2 and 53% (Paired t-test: t = 2.4, P = 0.2) . At day 4 post-treatment, there was a significant difference in thrips mortality between the two treatments 66.0 and 38.2 % (Paired t-test: t = 2.4, P = 0.02). However, there were no significant differences in mortality at day 5,

6 and 7 post-treatment between the fungus-treated semiochemical-baited device and fungus-treated device without semiochemical respectively 53.0 and 50.2 % (Paired t-test: t = 2.4, P = 0.4), 37.2 and 42.2 % (Paired t-test: t = 2.4, P = 0.7), 43.2 and 44.5% (Paired t-test: t = 2.4, P = 0.9) (Figure 8. 2).

The number of thrips recorded per plant 7 days post-inoculation was significantly lower in the *M. anisopliae*-treated devices, 6.6 ± 1.4 and 8.7 ± 1.7 adults/plant in fungus-treated device without semiochemical and fungus-treated semiochemical-baited device, respectively, than in the control (19.8 ± 2.6 adults/plant) (ANOVA: $F_{2,90}$ = 12.9, P = 0.0001). However, there was no significant difference between the thrips pathogen treatments (Figure 8. 3).

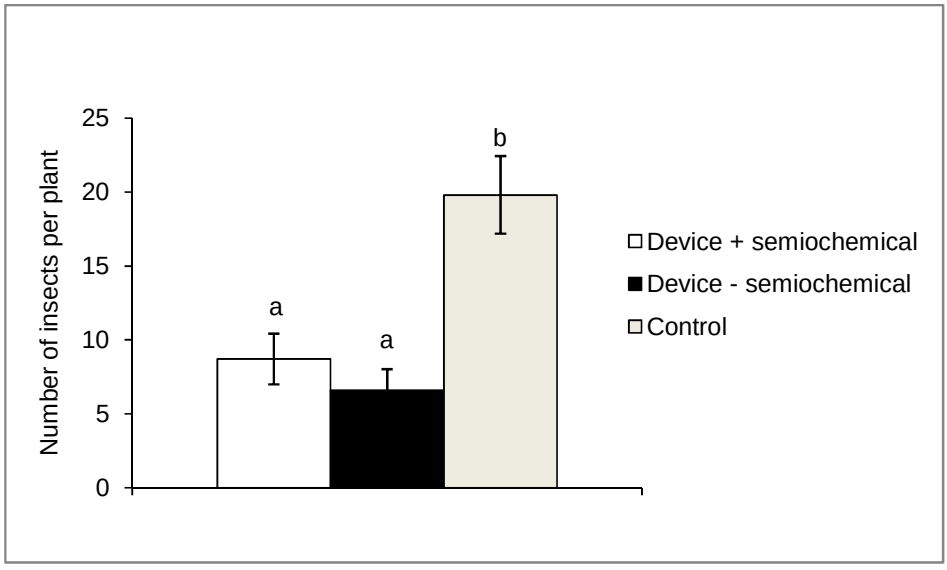


Figure 8. 3 : Number of *Frankliniella occidentalis* adults per plant from field cages sampled at 7 days after placement of an auto-inoculation device in field cages.

Conidial viability was not affected in *M. anisopliae*-treated device without semiochemical at 7 days after treatment. However, in *M. anisopliae*-treated semiochemical-baited device, conidial viability decreased from 80 to 6% at 2 and 7 days post-inoculation, respectively (ANOVA: $F_{1,42} = 2.3$, $P < 0.0001$) (Figure 8. 4).

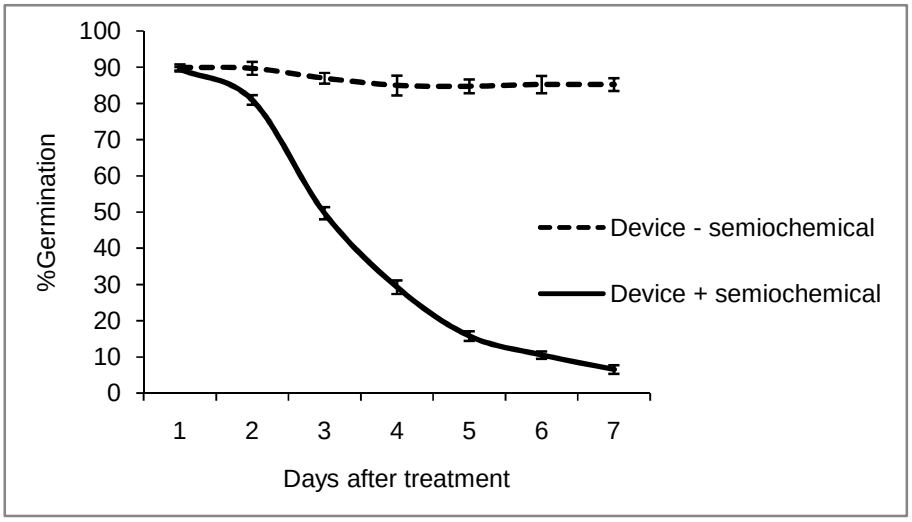


Figure 8. 4 : *Metarhizium anisopliae* conidial viability in the auto-inoculation device at 1 to 7 days after placement of an auto-inoculation in the field cage.

In the treatment device without semiochemical, a positive correlation was observed between temperature and conidial viability while humidity was negatively correlated with conidial viability, conidial acquisition and temperature (Table 8. 1). In semiochemical-baited device treatment, there was a positive correlation between mortality and conidial viability and a negative correlation between conidia acquisition and conidial persistence.

Table 8. 1: Pearson (R) correlation between parameters affecting thrips mortality of *Metarhizium anisopliae* in the field cages with and without semiochemical.

Device - semiochemical	Mortality	Persistence	C. acquisition	Temperature	Humidity
Mortality		-0.1	0.3	-0.01	-0.02
Persistence			-0.03	**0.6**	**-0.5**
C. acquisition				0.3	**-0.4**
Temperature					**-0.9**
Humidity					
Device + semiochemical					
Mortality		**0.6**	-0.3	0.2	-0.2
Persistence			**-0.7**	0.1	0.02
C. acquisition				-0.09	-0.05
Temperature					**-0.9**
Humidity					

In bold, significant values (except diagonal) at the level of significance alpha=0.050 (two-tailed test)

There was no correlation between thrips mortality and temperature neither was there correlation between mortality and humidity (Table 8. 1).

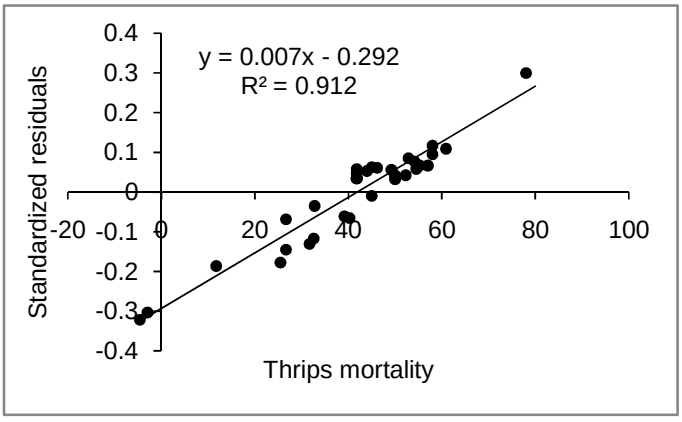


Figure 8. 5: Linear Regression model of mortality as affected by various parameters in treatment without semiochemical. Mortality = 0.49 × Conidial viability × $9.7e^{-05}$ × Conidia acquisition -0.5 × Temperature

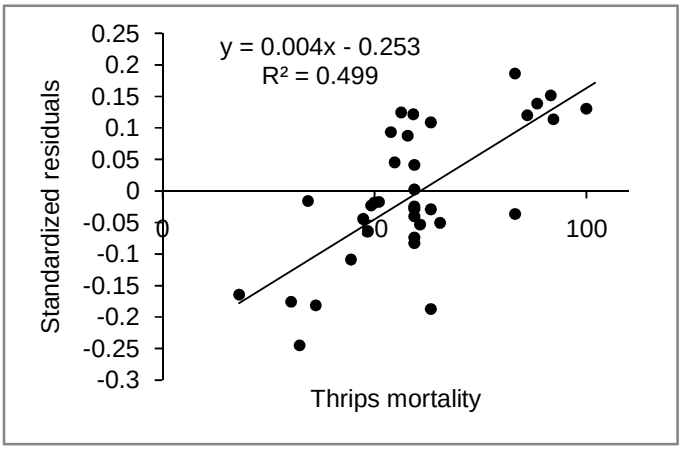


Figure 8. 6: Linear Regression model of mortality as affected by various parameters in treatment with semiochemical. Mortality = 0.5 × Conidial viability + 5.3 e^{-05} × Conidia acquisition + 1.4 × Temperature

A Linear Regression Model of mortality indicated that, conidial acquisition (Student's t = 2.05; P = 0.04) was the most relevant parameter in the fungus-treated device without semiochemical treatment (Figure 8. 6); whereas conidial viability was the most relevant parameter in the fungus-treated semiochemical-baited device treatment (Student's t = 4.24; P = 0.0001) and temperature (Student's t = 2.78; P = 0.007) (Figure 8. 5). The treatment without semiochemical (R^2 = 0.91) fitted better with the linear model than the treatment with semiochemical (R^2 = 0.49) (Figures 8. 5 and 8. 6).

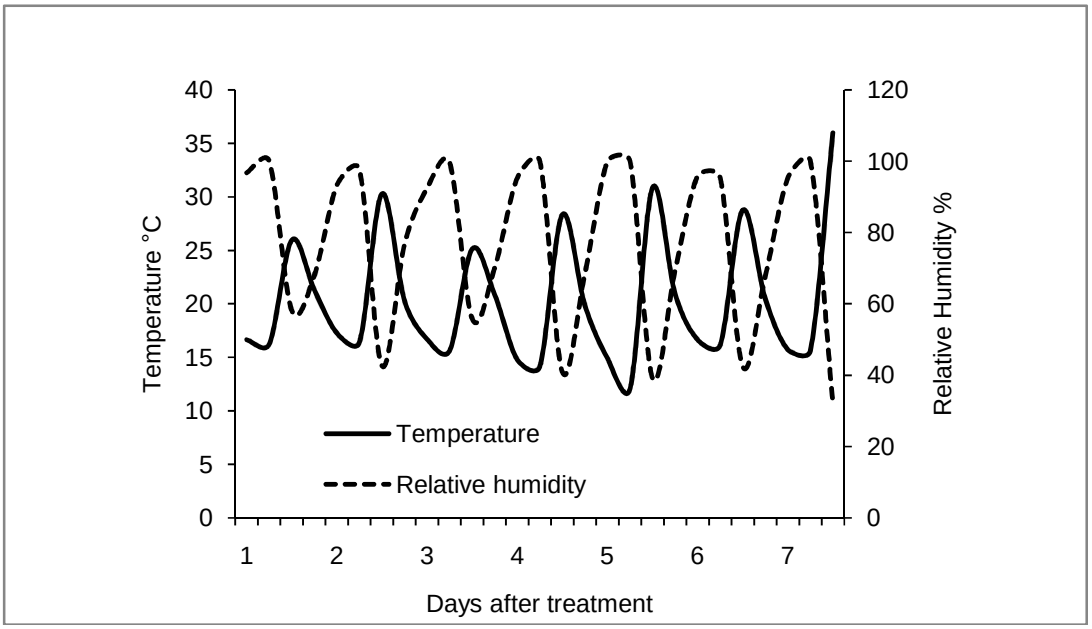


Figure 8. 7: Mean values of Temperature (°C) and Relative humidity (%) in field-cages during the experimental period

8.4 Discussion

Adult thrips were attracted to both fungus-treated device baited with or without semiochemical treatments. However, the attraction was significantly higher in the semiochemical-baited device than in the device without semiochemical. Increase in *F. occidentalis* catches due to the semiochemical LUREM-TR was previously reported by (Subramanian *et al*., 2009; Till *et al*., 2009). *Frankliniella occidentalis* in semiochemical-baited device treatment picked up significant amount of conidia as compared to the device without semiochemical. This could be attributed to frequent visits of the insects to semiochemical-baited device. Similar observations were reported by Migiro *et al*. (2010) with leaf-mining flies.

Frankliniella occidentalis mortality was significantly higher in the fungus-treated semiochemical-baited device treatment one day post-inoculation than in the device without semiochemical which could correlate to the high amount of conidia acquired by the insects. Although the concentration of the inoculum picked by single thrips increased over time (Figure 8. 1), this was not necessarily translated into increase in mortality. On the other hand, lower thrips mortality was obtained in the device without semiochemical treatment one day post-inoculation and could be explained by weak attraction of thrips to the device and subsequent low inoculum acquisition by the insects. Migiro *et al*. (2010) reported that mortality of leaf-mining flies was positively correlated with the amount of fungal conidia picked by the insect from devices contaminated by fungal entomopathogens and our results are in agreement with previous finding.

The lower mortality recorded in the *M. anisopliae*-treated semiochemical-baited device treatment at 6-7 (Figure 8. 2) days post-inoculation can be explained by reduced viability of conidia observed during the experimental periods from day 2 onward (Figure 8. 4) due to the negative effect of the semiochemical on conidial viability (Figure 8. 4). Complete inhibition of conidial germination was observed after two days following exposure of fungal culture to semiochemical in a desiccator (Niassy S. and Maniania N. K., unpublished). The main component of the LUREM-TR semiochemical is ethyl-isonicotinate (Teulon, pers. comm.), a pyridine compound, which has been reported to have antifungal properties (Soldatenkov and Kolyadina, 2001; Bordoloi *et al*., 2002; Wei and Mei, 2009). Contrary to semiochemical-baited device treatment, viability of conidia in the device without semiochemical treatment was not affected 7 days post-inoculation. Maniania (1998) reported that fungal inoculum can persist for 3 weeks within an autoinoculation device used for tsetse fly suppression in field conditions. Contrary to current device use for thrips suppression, the tsetse fly contamination

device largely utilizes visual cues and the urine odorants are clearly separated from the device with minimal if any impact on the conidia in the autoinoculator. Our results indicate that the close proximity of the fungal spores to the attractant and the volatility of the thrips attractant negatively impacted on the conidia hence low persistence of the insect pathogen. Although further modification are required in the "lure and kill" strategy, the reduction of thrips populations in both insect pathogen treatments, as compared to the control treatment, demonstrates the prospects of autoinoculation device strategy for the control of F. occidentalis, particularly in the screenhouses. The fact that we were able to observe mycosed thrips in the flowers in the two fungal treatments clearly shows that the insects were able to pick up inoculums from the device leading to infection and reduction in population on French beans in the field cages.

Correlations were observed between different parameters in both the semiochemical-baited device and device without semiochemical treatments (Table 8. 1). Positive correlation between mortality and conidial viability in semiochemical-baited device treatment confirmed the decrease in mortality of thrips from 2 days post-inoculation due to the apparent antifungal effect of the semiochemical, which was not the case in the device free of semiochemical.

Moreover, the negative correlation between relative humidity/temperature and conidial acquisition in the device without semiochemical can be explained by the fact that thrips were less active and subsequently acquired less inoculum when temperature was low (Figure 8.7). It has already been reported that certain climatic thresholds affect thrips activity (Rhainds *et al*., 2007; Alghali, 1991). The optimum temperature for high thrips number is 15-25 ℃ (Trdan *et al*., 2003; Boissot *et al*., 1998) which implies that thrips would pick more conidia at temperatures when they are more active.

In conclusion, the study demonstrated that autoinoculation can be used for control of thrips in screenhouses but this needs to be further demonstrated in the open field. Addition of semiochemical might have resulted in increase attraction of thrips to the device although the attractant appeared to negatively affected persistence of fungal conidia. There is therefore the need to screen for semiochemical that might be compatible with insect pathogen that could be further exploited for field application and suppression of thrips on vegetables.

CHAPTER NINE

9.0 SUBLETHAL CONCENTRATIONS OF *METARHIZIUM ANISOPLIAE* (METSCHNIKOFF) SOROKIN AFFECT FEEDING BY *THRIPS TABACI* LINDEMAN AND VECTOR COMPETENCE TO TRANSMIT IRIS YELLOW SPOT VIRUS (IYSV) ON ONION PLANT

9.1 Introduction

The onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), causes serious damage to crops such as onion and garlic (Lu and Lee, 1985) and is a major constraint in onion bulb production in the world (Khan *et al*, 2008; Waiganjo *et al*., 2006). *Thrips tabaci* feeds by piercing individual cells and sucking the contents. The cells lose their normal color, resulting to the leaf to whiten. In addition to direct damage by feeding, *T. tabaci* also causes indirect damage by transmitting pathogens, especially the tospovirus , Iris Yellow Spot Virus (IYSV) (Bunyaviridae: Tospovirus) (Zen *et al*., 2008; Jenser *et al*., 2001; Chatzivassiliou, 2001; Inoue *et al*., 2010). The incidence of IYSV diseases can reach 50-60%, resulting in heavy yield losses (Kritzman *et al*., 2001). Thrips only acquire tospovirus at the larval stage, especially first-instar (Nakahara and Monteiro, 1999). Once acquired tospoviruses propagate in their vector, viruliferous thrips are capable of spreading the virus for the rest of their lives (Ullman *et al*., 2002; Jones, 2005; Whitfield *et al.*, 2005).

Management of onion thrips heavily relies on synthetic chemical sprays (Waiganjo *et al*., 2006; Groves *et al*., 2001; Saxena *et al*., 1997; Chatzivassiliou, 2008). Although effective, the use of these synthetic chemical insecticides is no longer lucrative due to thrips resistance and other environment hazards (Morishita, 2008; Jensen, 2004). Alternatives to chemical

insecticides include cultural practices such as intercropping, use of resistant crop varieties, mass-trapping using semiochemicals (Trdan *et al*., 2005; Teulon *et al.*, 2010; Nderitu *et al.*, 2007; Groves *et al*., 2001). Biological control using entomopathogenic fungi is among the alternatives to synthetic chemical insecticides being considered among the pathogens (Butt and Brownbridge, 1997). For instance, Maniania *et al*. (2003) were able to achieve control of *T. tabaci* in onion crop using an isolate of *Metarhizium anisopliae* (Metschnikoff) Sorokin (Ascomycota: Hypocreales).

The efficacy of entomopathogenic fungi is generally measured in terms of virulence. However, infection by entomopathogenic fungi has been reported to affect feeding in crop pest insects (Ekesi *et al*., 2000; Migiro *et al*., 2011; Seyoum *et al*., 1994) and haematophagus insects such as mosquitoes (Fang *et al*., 2011; Howard *et al*., 2010; Mnyone *et al*., 2011; Scholte *et al.*, 2005; Ondiaka *et al.*, 2008); vector competence in parasite transmission in mosquitoes (Blanford *et al*., 2005; Fang *et al*., 2011). In the present study we therefore investigate whether infection of *T. tabaci* by *M. anisopliae* would affect their feeding and their competence to vector IYSV in onion plant.

9.2.0 Materials and methods

9.2.1 Insect colonies

Adults *T. tabaci* were obtained from the Mass Rearing Unit at the International Centre of Insect Physiology and Ecology (*icipe*). They were identified using the Lucid key (Moritz *et al.*, 2005). Around 500 adult *T. tabaci* were allowed to oviposite on fresh snow pea pods *Pisum sativum* L. that were previously soaked in 10% sucrose for two days after which pods were transferred into clean plastic rearing containers (9 cm diameter and 16.5 cm

height) with paper towel (11 × 11 cm) to allow pupation of larvae. The lid of the rearing container was perforated and covered with a thrips-proof mesh for aeration. Insects were reared at 25 ± 2 ºC, 60-80% RH, with a 12 L: 12 D photoperiod.

9.2.2 Host-plant

Onion plants *Allium cepa* L. were grown in a screenhouse (2.8 m length 1.8 width 2.2 m height) in 15 cm pots (5–8 plants per pot) using a mixture of compost manure/cow dung and clay soil in a ratio of 1:5, respectively. One-month old onion plants were used in the bioassays.

9.2.3 Virus

Virus-infected materials were obtained from onion plants showing severe symptoms of tospovirus disease from the botanical farm at *icipe*'s Duduville campus. The identity of IYSV was confirmed in laboratory using Agdia flash kit and IYSV-specific DAS-ELISA (USA). Infected leaves were cut into 3-4 cm pieces and were used to infect first-instar larvae of *T. tabaci.*

Plate 9. 1: Virus source from onion cultures infected with IYSV in pesticide free conditions at the icipe's botanic farm in Duduville.

9.2.4 Fungus

Metarhizium anisopliae isolate ICIPE 69 used in this study was obtained from the *icipe*'s Arthropod Germplasm Centre. It has already been reported to be virulent to *T. tabaci* (Maniania, 2003 #12). The fungus was cultured on Sabouraud Dextrose Agar (SDA) and incubated at 25 ± 2°C. Conidia were harvested by scrapping the surface using a spatula and were suspended in 10 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing glass beads. Conidial suspensions were vortexed for 5 min to produce homogenous suspensions. Conidial concentrations were determined using a haemocytometer. The viability of conidia was determined before any bioassay by spread-plating 0.1 ml of a 3×10^6 conidia ml^{-1} suspension onto 9-cm Petri dishes containing SDA medium. A sterile microscope cover slip was placed on each plate, and the plates were incubated in complete

darkness at 25 ± 2°C and examined after 18-20 h. Percentage germination of conidia was determined by assessing the number of germ tubes formed among 100 random conidia on the surface area covered by each cover slip under the light microscope (400 ×). Four replicates were used for each bioassay.

9.2.5 Infection with virus

Newly-emerged larvae of *T. tabaci* were transferred to rearing plastic jars (9 cm diameter and 16.5 cm height) containing 3-4 cm IYSV-infected onion leaves and were allowed to feed until pupation. Paper towel was lined at the bottom the jar and moistened daily to avoid leaves to dry. Insects were maintained as described above.

Plate 9. 2: IYSV infected onion leave piece used for inoculation of first instars larvae cohort of *Thrips tabaci.*

To confirm the presence of virus in adult thrips, groups of 5 IYSV-infected adult thrips were selected at random and sprayed with different concentrations of *M. anisopliae* as described earlier. They were then transferred to 9-cm Petri dish containing a virus-free 4-cm^2 leaf disc. Each group of the 5 thrips was transferred into 1.5-ml eppendorf tubes containing 100 µl of general extraction buffer (Agdia) and ground for 5 min. The extract was then loaded into 96-well plate. Plates were coated with specific IYSV antibody and incubated overnight at 4°C. Each sample was run in duplicate. Plates were thereafter incubated overnight at 4°C, covered in a plastic wrap in a humid box. Between each step, plates were washed five times with ELISA wash buffer (0.8 g of KH_2PO_4, 0.8 g of KCl, 4.6 g of $NaPO_4$ dibasic, 32 g of NaCl, and 2 ml of Tween in 4 liters of deionized water), pH 7.3. Following washing, 100 µl goat-anti-rabbit alkaline phosphatase diluted conjugate was added to each well, and plates were incubated for 2 hours at room temperature after which they were washed three times, followed by addition of 100 µl substrate [(1 mg/ml of p-nitrophenyl phosphate), Agdia Co.]. Plates were incubated at room temperature for 1 hour in the dark to allow colour development and the reaction was stopped using 1M NaOH. Viral titers in the samples were determined by reading absorbance at 405 nm (A_{405}) using an ELISA plate reader (Epoch™, Gen5™800 Biotek). Thirteen (13) of 500 individual cohorts were used in the whole experiment.

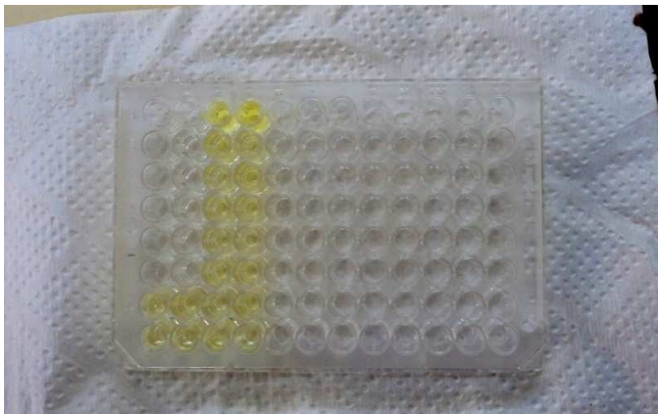


Plate 9. 3: 96-wells plate showing reactions using the DAS-ELISA technique

9.2.6 Inoculation of insect with fungus

Five (5) viruliferous thrips were placed in a 9-cm Petri dish lined with a filter paper and were directly sprayed with three concentrations of *M. anisopliae* (1×10^5, 1×10^6 and 1×10^7 conidia ml^{-1}) using a Burgerjon (1956) spray tower. Clean fresh onion leaf discs (4 cm^2) were then introduced in the Petri dishes and served as food. The three concentrations corresponded to sublethal and 50% lethal concentration (LC_{50}), 1×10^5, 1×10^6 and 1×10^7 conidia ml^{-1}, respectively. In the control treatments, insects were treated with sterile distilled water containing 0.05 % Triton X-100. Test-insects were incubated at 25 ± 2 °C and 90 ± 2 % RH with a photoperiod of 12 h light: 12 h dark.

9.2.7 Effect of fungal infection on IYSV-infected *T. tabaci* feeding punctures

To assess the effect of fungal infection on feeding, the number of feeding punctures was counted daily for 5 days on a 4-cm^2 leaf disc using a dissecting microscope. Treatments were randomized and experiment repeated five times with five replicates.

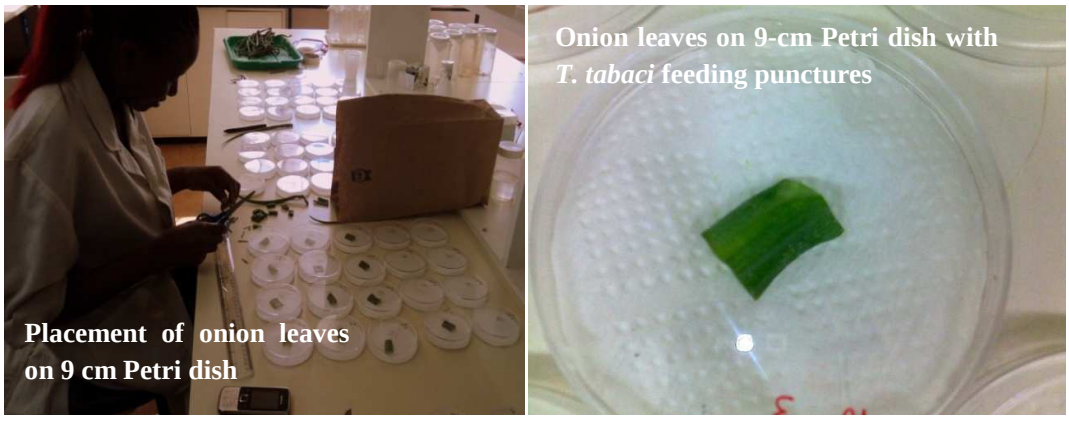


Plate 9. 4: Experimental design and 9-cm Petri dish containing 5 viruliferous *Thrips tabaci* treated with *Metarhizium anisopliae* and 4 cm^2 onion leave with feeding punctures

9.2.8 Effect of *Metarhizium anisopliae* infection on IYSV-infected *Thrips tabaci* competence to vector virus on onion plant

Group of five IYSV-infected thrips were sprayed with different concentrations of *M. anisopliae* as described earlier. They were then transferred on 4-cm^2 virus-free onion leaf discs in 9-cm Petri dish and allowed to feed for 24h. The experiment was carried out with one to 5-day old fungus-infected viruliferous thrips to assess the effect of the development of fungal infection in the insect on the transmission of the virus. In the control insects were sprayed with distilled water. Leaf discs were transferred into 1.5-ml Eppendorf tube and ground in 100 µl of general extraction buffer (Agdia) and processed as described earlier. The titer of the virus was determined using the procedure described above. Thirteen cohorts were used in the whole experiment.

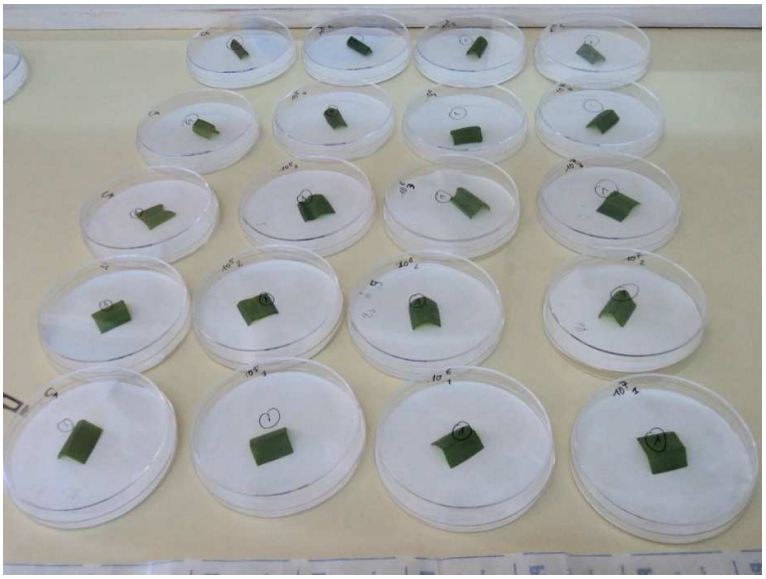


Plate 9. 5: Experimental design of a single replicate of feeding behaviour assessment of 5 IYSV infected *Thrips tabaci* treated with *Metarhizium anisopliae* concentrations placed on 9-cm Petri dish with virus free onion leaves.

9.2.9 Data analysis

Data were arcsine normalized before being subjected to analysis of variance (ANOVA) using a general linear model (GLM). Means were separated using Student-Newman-Keuls (SNK). Multiple comparisons between treatments were performed and interactions between factors (cohorts, concentrations and days) were also done using ANOVA. A Pearson correlation was performed to determine the relationship between feeding punctures, IYSV titer and IYSV transmission. In all analysis the level of significance, was kept at 95% accuracy.

9.3 Results

9.3.1 Evidence of acquisition of IYSV by adult thrips following infection of larvae

DAS ELISA readings at 405 nm showed that positive control values varied between 2.5 and 3.8 while the negative control ranged between 0.04-0.07. Exposure of larvae to IYSV-infected onion leaves resulted in infection of adult thrips as illustrated by Figure 9. 1.

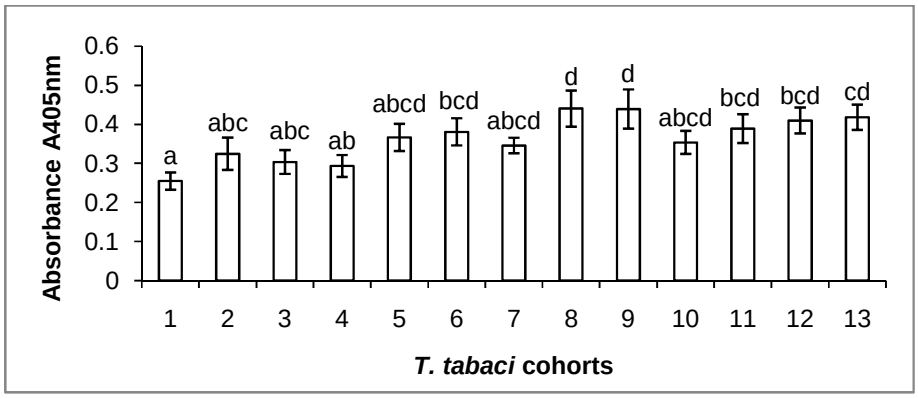


Figure 9. 1: Acquisition of *Thrips tabaci* cohorts reared on IYSV infected onion-leaves

Although the mean average of virus titers varied significantly between the cohorts (ANOVA: $F_{12, 240} = 4.6$; 0.0001) (Figure 9. 1). No significant differences were observed between the control and the fungal concentrations (ANOVA: $F_{3, 240} = 0.4$; 0.7).

9.3.2 Effect of *Metarhizium anisopliae* infection on IYSV-infected adult *Thrips tabaci* on feeding punctures

The overall number of feeding punctures/4-cm^{-2} leaf disc was 64.2 ± 1.0, 54.0 ± 0.7, 40.0 ± 0.4 and 22.6 ± 0.4 in the control, 10^5, 10^6 and 10^7 conidia ml^{-1} concentrations of *M. anisopliae*, respectively, which was significantly different between the treatments (ANOVA: $F_{3, 480} = 50.4$; $P < 0.0001$). The higher concentration of *M. anisopliae* (1×10^7 conidia ml^{-1}) had the least feeding punctures between the three fungal concentrations.

Table 9. 1: Cumulative mean number of feeding punctures by IYSV-infected *Thrips tabaci* on onion leaves following infection by *Metarhizium anisopliae.*

	***Metarhizium anisopliae* treatments conidia ml^{-1}**			
Days after fungal treatment	**Control**	**1×10^5**	**1×10^6**	**1×10^7**
2	87.0 ± 5.8Aa	88.0 ± 5.8Aa	89.0 ± 5.8Aa	90.0 ± 5.8Aa
3	149.6 ± 8.3Ab	148.2 ± 8.1Ab	128.9 ± 7.3ABa	115.9 ± 7.4BCa
4	218.3 ± 10.8Ac	202.0 ± 9.4ABc	174.2 ± 9.5BCb	140.9 ± 8.8Cb
5	289.4 ±13.5Ad	259.0 ± 11.2Ad	213.3 ±12.1Bc	164.3 ± 10.8Cbc
6	350.3 ± 18.0Ae	313.2 ± 12.8Be	255.5 ± 16.0Cd	186.1 ± 12.9Dc

Means within the same row followed by the same capital letters are not significantly different by SNK
Means within the same column followed by the same small letter are not significantly different by SNK.

There were also significant differences in feeding punctures between days post-infection (ANOVA: $F_{4,480} = 182.9$; $P < 0.001$). The interaction between *M. anisopliae* concentrations and days was also significant (ANOVA: $F_{12,480} = 7.0$; $P < 0.0001$) (Table 9. 2).

9.3.3 Effect of fungal infection on vector competence of IYSV-infected adult *Thrips tabaci* to transmit virus on onion leaves

IYSV-infected adult thrips effectively transmitted virus to onion leaves (Figure 9. 2). However, the mean average of virus titers varied significantly between the cohorts (ANOVA: $F_{12, 240} = 2.4$, $P = 0.005$) and the treatments (ANOVA: $F_{3,240} = 14.4$, $P = 0.0001$) (Figure 9. 2). For example, IYSV titer was higher in the control (0.481) than in fungus-treatments (0.388, 0.372 and 0.320 at 10^5, 10^6 and 10^7 conidia ml^{-1}, respectively) 5 days post-infection.

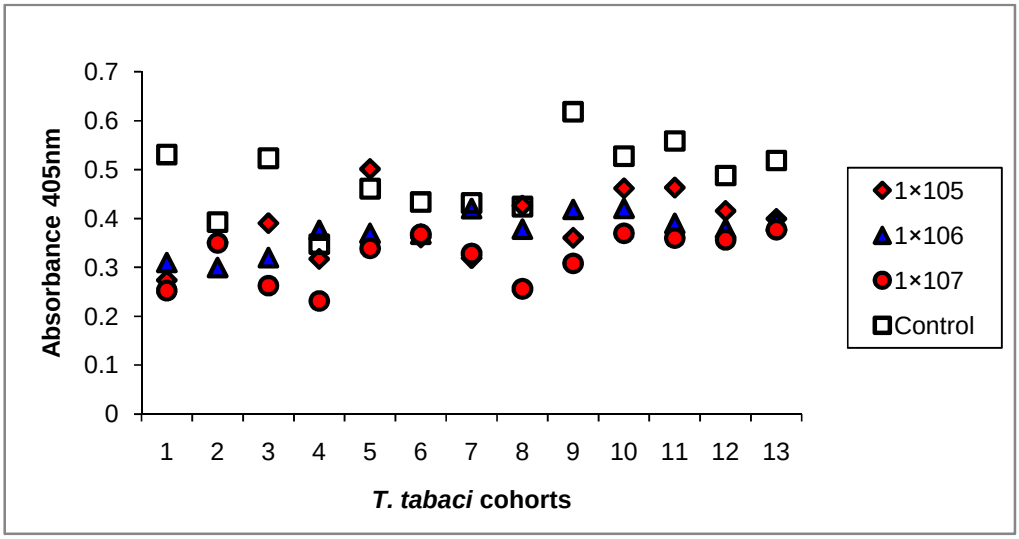


Figure 9. 2: Effect of *Metarhizium anisopliae* concentrations on IYSV-infected *Thrips tabaci* cohorts transmission on onion leaves

Days post-inoculation did not have significant effect on transmission of the virus (ANOVA: $F_{4,240} = 1.4$, P = 0.2) (Figure 9. 3). For example at day 1 post-inoculation, the virus titer was 0.510, 0.389, 0.366 and 0.315 in the control, 10^5, 10^6 and 10^7 conidia ml^{-1}, respectively; while at day 5 post-infection the titer was 0.459, 0.381, 0.360 and 0,331 in the control, 10^5, 10^6 and 10^7 conidia ml^{-1}, respectively. The interaction between *M. anisopliae* concentrations and time was not significant either (ANOVA: $F_{12,240} = 1.3$, P = 0.2) (Figure 9. 3).

There was a negative correlation between IYSV titer in adult *T. tabaci* and IYSV transmission on onion leaves; while there was a positive correlation between feeding punctures and IYSV transmission. However no correlation was found between *T. tabaci* feeding punctures and IYSV virus titers (Table 9. 3).

Table 9. 2: A Pearson correlation (R, P) between *Thrips tabaci* feeding punctures and IYSV titer in adult thrips and transmission on onion leaves

	Feeding punctures	**IYSV titer**	**Transmission**
Feeding punctures		- 0.012, 0.8	**0.2, 0.03**
IYSV titer			**- 0.2, 0.02**
Transmission			

In bold, significant values at the level of significance alpha=0.05 (two-tailed test)

9.4 Discussion

First-instar larvae of *T. tabaci* reared on IYSV-infected leaves successfully acquired the virus, which was later detected in adult insects. However, acquisition of the virus was not uniform since significant differences in virus titer were observed between the cohorts. The

difference in the concentration of virus within the same onion leaf is supported by the study of (Kritzman *et al*., 2001) who found that the IYSV was not evenly distributed within infected onion leaf.

Fungal infection by *M. anisopliae* significantly affected the feeding by adult *T. tabaci* by reducing the number of punctures. These results are in agreement with previous published works (Fargues *et al*., 1994; Moore *et al*., 1992; Seyoum *et al*., 1994; Migiro *et al*., 2011). Furthermore, feeding punctures were significantly reduced according to fungal concentrations as reported by Migiro *et al.* (2011). The higher the concentration was the least feeding punctures were recorded. Stafford *et al.* (2011) reported an increase in feeding by *Frankliniella occidentalis* infected with plant pathogen Tomato Spot Wilt Virus, but this was not observed in the present study. Birithia (pers. communic.) did not also observed any difference in feeding between IYSV-infected and IYSV-free *T. tabaci.*

Metarhizium anisopliae-infected adult thrips transmitted lower titers of virus than did the control. The transmission of the pathogen can occur in different ways; but the mechanism in which the fungal infection interferes with transmission of the virus is still unclear. The positive correlation between the feeding punctures and the transmission of the virus in the onion leaves could imply that the transmission is through feeding. Similar observations were made by Groves *et al*. (2001) who found that application of imidacloprid on *Frankliniella fusca* resulted in reduction of probing and thus, transmission of tospovirus. The effect of fungal infection on pathogen transmission is well studied in mosquitoes. For instance, Fang *et al*. (2011) reported that *M. anisopliae* expressing salivary gland and midgut peptide 1 (SM1) *Anopheles gambiae* interfered with *Plasmodium falciparum* sporozoites attachment to salivary glands. The negative correlation between IYSV titer in adult *T. tabaci* and its transmission on onion leaves suggests on one hand the possibility of viral replication in adult *T. tabaci* as reported by Inoue *et al.* (2010); and on the other hand, these results suggest the

possibility of *M. anisopliae*-infected thrips to refrain them from transmitting IYSV to onion leaves.

These results are the first report on the interaction between *M. anisopliae* and tospovirus transmission. Our results suggest that *M. anisopliae* application at sublethal concentrations can significantly contribute to tospovirus reduction in the field. However, further investigations are required to determine the effect of *M. anisopliae* on IYSV acquisition by *T. tabaci* larvae.

CHAPTER TEN

10.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

10.1 General discussion

Frankliniella occidentalis is considered to be a major pest of vegetable and ornamental crops: French bean, tomato, onion, chrysanthemum cowpea. Outbreaks of WFT can lead to considerable losses and crop rejections by costumers. In addition to the cosmetic damage they cause, WFT is also a vector of tospovirus which often mislead farmers to use fungicide to control secondary pathogen diseases.

Management of thrips is mostly based on the large-scale application of synthetic chemical pesticides. The use of these synthetic chemical pesticides pollutes the environment by indiscriminately suppressing thrips and other non-target organisms, contamination of the produce, and toxicity to users and consumers.

The use of semiochemical and blue sticky traps for mass trapping of WFT, intercropping and cultural practices are among the alternatives being considered to avoid heavy damages by farmers.

For all theses constraints aforementioned, researches for environmentally safe control strategy have been promoted for thrips control. Among many control strategies, entomopathogenic have appeared as one of the most promising tool to contain thrips without compromising ecological balance and food quality. The most commonly investigated entomopathogenic fungi in Africa is the specie *Metarhizium anisopliae,* as they have a wide geographic spread and host range.

These entomopathogenic fungi have been widely considered for biological control of agricultural pests.

Many aspects of the use of entomopathogenic fungi for thrips control have been investigated, for instance identification of virulent isolates, effect of temperature on fungal virulence, effect of host plant. However there are other important parameters crucial for biopesticide developmentsdevelopments which were simply overlooked. These includes appropriate bioassays for strain selection involving spore production and molecular characterization, compatibility with other IPM tools i. e agrochemicals or the association of entomopathogenic fungi with semiochemicals, to improve fungal application efficiency and sustainability attractant-infection and autodissimination strategy for the control of Thrips.

The current study was initiated to develop a fungal based product for the control of *F. occidentalis* by providing an original isolate that is virulent to WFT both adult and larval stages, easily mass producible with a wide host range.

This study has demonstrated that ICIPE 69 is the best isolate for thrips control both adult and larval stages. The compatibility of this isolate of *M. anisopliae* with imidacloprid and thiamethoxam which is a huge advantage to farmers since most exported crops are host of a wide range diversity of pest. On the other hand, fungicides, chloropyrifos and neem were not appropriate candidates to be combined with *M. anisopliae* for thrips control.

The present study also established that *M. anisopliae* can be utilized in an autoinoculative technique to deliver conidia to thrips species. Thrips attractant such as Semiochemical and colour baited traps can boost thrips conidial acquisition leading to higher mortalities. However, there is a crucial need to identify a compatible attractant that doesn’t affect fungal viability within the device. The present study revealed that combined with semiochemicals, the device was effective in attracting and infecting *M. anisopliae* conidia resulting in high mortality of the thrips the 2-3 first days. Previous researches were more focused on

inundative sprays of fungal suspension whereby conidial persistence was challenged. In our study conidial persistence was constant in the device semiochemical free for more than 10 days after application. This approach is more cost effective as compared to inundative sprays and less time consuming in term of thrips control. Secondary infections are advantages that may be expected by using such application technique.

The study showed that the use of *M. anisopliae* application at sublethal concentrations can significantly contribute to tospovirus reduction in the field, which is a hugh advantage as compared to chemicals.

ICIPE 69 is actually under registration for commercialization in Kenya and east Africa. Its pathogenicity to various thrips species such as *Megalurothrips sjostedti*, *Thrips tabaci*, and *Frankliniella occidentalis* has been already demonstrated. Its use for thrips control can significantly reduce thrips damage and therefore open new possibility for exports safe crops.

10.2 Conclusions

With regard to the results obtained in the laboratory, it was concluded that:

1. ICIPE 69 is the best *M. anisopliae* isolate for the control of *F. occidentalis*.

2. ICIPE 69 was compatible with agrochemicals like Imidacloprid, Thiamethoxam and abamectin while fungides, neem and chlorpyrifos were toxic.

3. Autoinoculative technique can be a promising technique that can be used for thrips control.

4. Fungal application can contribute significantly in the control of Tospovirus diseases

10.3 Recommendations

In the course of this study, various questions arose, which may be subject for future studies:

1. ICIPE 69 used in this study originated from DRC Congo. Further molecular studies should be undertaken to study the genome of this isolate.

2. ICIPE 69 seems to be highly virulent on thrips species and less virulent on termites, studies should be conducted to understand the specificity of that isolate on thrips species.

3. A field evaluation of the auto inoculative technique should be conducted in order to identify a cost effective strategy for thrips control using spot sprays

4. Other potential semiochemical which are compatible to *M. anisopliae* should be screened and identified for being used in an autoinoculative technique.

REFERENCES

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

Alghali, A. M. 1991. The effects of some agrometeorological factors on the fluctuation of flower thrips, *Megalurothrips sjostedti* Trybom (Thysanoptera: Thripidae), on two cowpea varieties. *Journal of Plant Protection in the Tropics*, **8**: 63-68.

Alizadeh, A., Samih, M. A., Khezri, M., Riseh, S. R. 2007. Compatibility of *Beauveria bassiana* (Balsamo) Vuillemin with several pesticides. *International Journal of Agriculture and Biology*,**9**: 31–34.

Arakane, Y. and Muthukrishnan, S. 2010. Insect chitinase and chitinase-like proteins. *Cellular and Molecular Life Science*, **67**: 201-216.

Aramburu, J., Rodriguez, M., Ariño, J. 2000. Effect of Tomato Spotted Wilt Tospovirus (TSWV) Infection on the Fruits of Tomato "Lycopersicon esculentum" Plants of Cultivars Carrying the SW-5 gene. *Journal of Phytopathology* **148**: 569-574.

Baker, C. R. B., Barker, I., Bartlett, P. W., Writh, D. M. 1993. Western flower thrips, its introduction and spread in Europe and role as a vector of Tomato Wilt Virus in **Ebbels D**, Plant Health and the European Single Market **(ed.)** British Crop Protection Council, Farnham, pp. 355-360.

Baker, G.L., Specht, A. C. Lodge, K. J. 2009. Chitinases are essential for sexual development but not vegetative growth in *Cryptococcus neoformans*. *Eukaryotic Cell*, 1692-1705.

Baratto, C. M., da Silva, M. V., Santi, L., Passaglia, L., Schrank, I. S., Vainstein, M. H. Schrank, A. 2003. Expression and characterization of the 42 kDa chitinase of the biocontrol fungus *Metarhizium anisopliae* in *Escherichia coli*. *Canadian Journal of Microbiology*, **49**: 723-726.

Baratto, C. M., Dutra, V., Boldo, J. T., Leiria, L. B., Vainstein, M. H. Schrank, A. 2006. Isolation, characterization, and transcriptional analysis of the chitinase chi2 gene (DQ011663) from the biocontrol fungus *Metarhizium anisopliae* var. *anisopliae*. *Current Microbiology*, **53**: 217-221.

Barreto, C.C., Staats, C. C., Schrank, A., Vainstein, M. H. 2004. Distribution of chitinases in the entomopathogen *Metarhizium anisopliae* and effect of N-Acetylglucosamine in protein secretion. *Current Microbiology*, **48**: 102-107.

Bennison, J., Maulden, K., Dewhirst, S., Pow, E., Slatter, P., Wadhams, L. 2001. Towards the development of a push-pull strategy for improving biological control of western flower thrips on chrysanthemum: Thrips and Tospoviruses, Proceedings of the 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound), Reggio Calabria, Italy, pp. 199-206.

Berry, N., Butler, R. C. Teulon, D. A. J. 2010. Responses of New Zealand flower thrips (*Thrips obscuratus*) to synthetic and natural stimuli (odour and colour) in a wind tunnel. *New Zealand Journal of Crop and Horticultural Science,* **34**: 121-129.

Blaeser, P., Lleonarti, M. S. and Sengonca, C. 2002. Laboratory studies on the development, longevity and reproduction of four Amblyseius predatory mites fed with *Tetranychus urticae* Koch (Acari: Tetranychidae) and *Franklinielia occidentalis* (Pergande) (Thysanoptera: Thripidae). *Nachrichtenblatt des Deutschen Pflanzenschutzdienstes* **54**: 307-311.

Blanford, S., Chan, B. H. K., Jenkins, N., Sim, D., Turner, R. J., Read, A. F., Thomas, M. B. 2005. Fungal pathogen reduces potential for malaria transmission. *Science,* **308**: 1638-1641.

Bogo, M. R., Rota, C. A., Pinto H. J., Ocampos, M., Correa, C. T., Vainstein, M. H., Schrank, A. 1998. A Chitinase Encoding Gene (chit1 Gene) from the Entomopathogen *Metarhizium anisopliae*: Isolation and Characterization of Genomic and Full-Length cDNA. *Current Microbiology,* **37**: 221-225.

Boissot, N., Reynaud, B., Letourmy, P. 1998. Temporal analysis of western flower thrips (Thysanoptera: Thripidae) population dynamics on Reunion Island. *Environmental Entomology,* **27**: 1437-1443.

Boldo, J. T., do Amaral, K. B., Junges, A., Pinto, P.M., Staats, C. C., Vainstein, M. H., Schrank, A. 2010. Evidence of alternative splicing of the chi2 chitinase gene from *Metarhizium anisopliae*. *Gene,* **462**: 1-7.

Boldo, J. T., Junges, A., do Amaral, K. B., Staats, C. C., Vainstein, M. H., Schrank, A. 2009. Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*. *Current Genetics,* **55**: 551-560.

Bordoloi, G. N., Kumari, B., Guha, A., Thakur, D., Bordoloi, M., Roy, M. K. Bora, T. C. 2002. Potential of a novel antibiotic, 2-methylheptyl isonicotinate, as a biocontrol agent against fusarial wilt of crucifers. *Pest Management Science,* **58**: 297-302.

Borgio, J. F., Bency, B. J., Sharma, N. 2008. Compatibility of *Metarhizium anisopliae* (Metsch.) Sorok. with *Ocimum sanctum* Linn. (Tulsi) (Lamiaceae) Extracts. *Ethnobotanical Leaflets*, **12:** 698-704.

Bradley, C. A., Lord, J. C., Jaronski, S. T., Gill, S. A., Dreves, A. J., Murphy, B. C. 1998. Mycoinsecticides in thrips management: Pests and Diseases (ed. by IOBC), pp. 177-182.

Broughton, S. and Herron, G. A. 2009. Potential New Insecticides for the Control of Western Flower Thrips (Thysanoptera: Thripidae) on Sweet Pepper, Tomato, and Lettuce. *Journal of Economic Entomology*, **102**: 646-651.

Bugeme, D. M., Knapp, M., Boga, H. I., Wanjoya, A. K. Maniania, N. K. 2009. Influence of temperature on virulence of fungal isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the two-spotted spider mite *Tetranychus urticae*. *Mycopathologia*, **167**: 221-227.

Burgejon, A. 1956. Pulverisation de poudrage au laboratoire par des preparations pathogens insecticides. *Annals of Epiphytology*, **4**: 677- 688.

Butt, T. M. and Brownbridge, M. 1997. Fungal pathogens of thrips: Thrips as Crop Pests (ed. by T Lewis) CAB International, Wallingford, UK, pp. 399-433.

Butt, T. M. and Goettel, M. S. 2000. Bioassays of entomopathogenic Microbes: by Navon and Aschers) CABI Internatinal Press, Wallinford, UK, pp. 141-195.

Chandler, D., Davidson, G., Grant, W. P., Greaves, J., Tatchell, G. M. 2008. Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. *Trends in Food Science & Technology*, **19:** 275-283.

Chatzivassiliou E.K. 2001. *Thrips tabaci*: an ambiguous vector of TSWV in perspective: Thrips and Tospovirus: Proceedings of The 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound).

Chatzivassiliou, E. K. 2008. Management of the spread of Tomato Spotted Wilt virus in tobacco crops with insecticides based on estimates of thrips infestation and virus incidence. *Plant Diseases*, **92:** 1012-1020.

Chen, T-Y, Chu, C-C., Henneberry, T. J. 2004b. Catching thrips (Thysanoptera: Thripidae) with various traps: Beltwide Cotton conferences (ed. by ET Natwick) USDA-ARS, WCRL Phoenix, A Z University of California Holtville, CA San Antonio TX, pp. 1703-1706.

Chen, T-Y., Chu, C-C., Fitzerald, G., Natwick, E. T., Henneberry, T. J. 2004a. Trap evaluations for thrips (Thysanoptera: Thripidae) and hoverflies (Diptera: Syrphidae). *Environmental Entomology*. **33**: 1416-1420.

Cuthbertson, A. G. S., Walters, K. F. A., Deppe, C. 2005. Compatibility of the entomopathogenic fungus *Lecanicillium muscarium* and insecticides for eradication of sweetpotato whitefly, *Bemisia tabaci*. *Mycopathologia*, 160: 35-41.

Danfa, A. and van der Valk, H. 1999. Laboratory testing of *Metarhizium* spp. and *Beauveria bassiana* on Sahelian non-target arthropods. *Biocontrol Science and Technology*, **9**: 187-198.

Dara, S. K. and Hountondji, F. C. C. 2001. Effects of Formulated Imidacloprid on Two Mite Pathogens, *Neozygites floridana* (Zygomycotina: Zygomycetes) and *Hirsutella thompsonii* (Deuteromycotina: Hyphomycetes). *Insect Science and Its Application*, **21:** 133 -138.

Davidson M. M., Butler, R. C., Winkler, S. Teulon, D. A. J. 2007. Pyridine compounds increase trap capture of *Frankliniella occidentalis* (Pergande) in a covered in a covered crop. *New Zealand Plant Protection*, **60:** 56-60.

Depieri, R. A., Martinez, S. S. Menezes, A. O. J. R. 2005. Compatibility of the Fungus *Beauveria Bassiana* (Bals.) Vuill. (Deuteromycetes) with Extracts of Neem Seeds and Leaves and the Emulsible Oil. *Neotropical Entomology*, **34**: 601-606.

Dimbi, S., Maniania, N. K., Lux, S.A., Ekesi, S. Mueke, J. M. 2003. Pathogenicity of *Metarhizium anisopliae* (Metsch.) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin to three adult fruit fly species: *Ceratitis capitata* (Weidemann), *C. rosa* var. *fasciventris* Karsch and *C. cosyra* (Walker) (Diptera: Tephritidae). *Mycopathologia* **156**: 375-382.

Dowd, P. F. and Vega, F. E. 1995. Autoinoculation device for contaminating insect with active agents, Vol. 5452540: by US Patent) Secretary of Agriculture Washington DC, The United States of America.

Down, R. E, Cuthbertson, A. G. S., Mathers, J. J., Walters, K. F. A. 2009. Dissemination of the entomopathogenic fungi, *Lecanicillium longisporum* and L. muscarium, by the predatory bug, *Orius laevigatus*, to provide concurrent control of *Myzus persicae*, *Frankliniella occidentalis* and *Bemisia tabaci*. *Biological Control*, **50**: 172–178.

Doyle, J. J. and Doyle, J. L. 1990. Isolation of plant of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Drummond, A., Ashton, B., Buxton, S. 2011. Geneious v5.4, Available from http://www.geneious.com/.

Ekesi, S. and Maniania, N. K. 2000a. *Metarhizium anisopliae*: An effective biological control agent for the management of thrips in horti and floriculture in Africa: Advances in Microbial Control of Insects Pests (ed. by RK Upadhyay) Kluwer Academic/Plenum publishers, New York.

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

Ekesi, S. and Maniania, N. K. 2007. Use of entomopathogenic fungi in biological pest management. Research Signpost Publishers, Trivandrum, Kerala, India.

Ekesi, S. Maniania. N. K., Ampong-Nyarko, K. 1999. Effect of Temperature on Germination, Radial Growth and Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Science and Technology*, **9**: 177-185.

Ekesi, S., Dimbi, S., Maniania, N. K. 2007. The role of entomopathogenic fungi in the integrated management of fruit flies (Diptera: Tephritidae) with emphasis on species occurring in Africa: Use of entomopathogenic fungi in biological pest management (ed. by S Ekesi & NK Maniania) Research Signpost Publishers, Trivandrum, Kerala, India, pp. 239-274.

Ekesi, S., Maniania, N. K., Lwande, W. 2000. Susceptibility of the legume flower thrips to *Metarhizium anisopliae* on different varieties of cowpea. *Biocontrol*, **45**: 79-95.

Ekesi, S., Maniania, N. K., Onu, I., Lohr, B. 1998. Pathogenicity of entomopathogenic fungi (Hyphomycetes) to the legumes flower thrips, *Megalurothrips sjostedti* (Thysanoptera Thripidae). *Journal of Applied Entomology*, **122**: 629-634.

El-Sayed, A. M., Mitchell, V. J., McLaren, G. F., Manning, L. M., Bunn, B., Suckling, D. M. 2009. Attraction of New Zealand Flower Thrips, *Thrips obscuratus*, to cis-Jasmone, a volatile identified from japanese honeysuckle flowers. *Journal of Chemical Ecology*. **35**: 656–663.

Enkerli, J., Ghormade, V., Oulevey, C., Widmer, F. 2009. PCR–RFLP analysis of chitinase genes enables efficient genotyping of *Metarhizium anisopliae* var. *anisopliae*. *Journal of Invertebrate Pathology* **102:** 185-188.

EPPO 2002. Diagnostic protocols for regulated pests *Frankliniella occidentalis. Builletin European and Mediterranean Plant Protection Organization*, **2:** 281–292.

Fang, W., Pei, Y. Bidochka, J. M. 2007. A regulator of a G protein signalling (RGS) gene, cag8, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. *Microbiology*, **153**: 1017-1025.

Fang, W., Vega-Rodríguez, J., Ghosh, A. K., Jacobs-Lorena, M., Kang, A., St-Leger, R. J. 2011. Development of transgenic fungi that kill human malaria parasites in mosquitoes. *Science*, 331: 1074-1077.

Fargues, J., Delmas, J. C., Lebrun, R. A. 1994. Leaf consumption by larvae of the Colorado potato beetle (Coleoptera: Chrysomelidae) infected with the entomopathogen, *Beauveria bassiana*. *Journal of Economic Entomology*, **87**: 67-71.

Freed, S., Jin, F-L., Ren, S-X. 2011. Determination of genetic variability among the isolates of *Metarhizium anisopliae* var. *anisopliae* from different geographical origins. World Journal of *Microbiological Biotechnology*, **27**: 359-370.

Funderburk, J. 2001. Ecology of thrips: Thrips and Tospoviruses, Proceedings of the 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound), Reggio Calabria, Italy, pp. 121-128.

Gabarra, R. 1995. The mirid bug *Dicyphus tamaninii* as a greenhouse whitefly and western flower thrips predator on cucumber. *Biocontrol Science and Technology*, **5**: 475-488.

Gao, Q., Jin, K., Ying, S-H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X-Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S, Zhong, Y., Ma, L-J., St-Leger, R. J., Zhao, G-P., Pei, Y., Feng, M-G., Xia, Y., Wang. C. 2011 Genome Sequencing and Comparative Transcriptomics of the Model Entomopathogenic Fungi *Metarhizium anisopliae* and *M. acridum. PLoS Genetic*, 7.

Gimenez-Pecci M., Bogo, McR., Santi, L., de Moraes, C. K., Correa, C. T., Vainstein, M. H., Schrank, A. 2002. Characterization of Mycoviruses and Analyses of Chitinase Secretion in the Biocontrol Fungus *Metarhizium anisopliae*. *Current Microbiology*, **45**: 334-339.

Gitonga, L. M. 2008. Seasonality and evaluation of parasitoid *Ceranisus menes* for biocontrol of thrips on French beans. *Journal of Agriculture Science and Technology*, **10**: 56-69.

Gitonga, L. M., Lohr, B., Overholt, W. A., Magambo, J. K., Mueke, J. M. 2002. Temperature-dependent development of *Megalurothrips sjostedti* and *Frankliniella occidentalis* (Thysanoptera: Thripidae). *African Entomology*, **10**: 325-331.

Goettel, M. S. and Inglis G. D. 1997. Fungi: Hyphomycetes: Manual of techniques in insect pathology (ed. by L Lacey) Academic, San Diego, CA, pp. 213-248.

Gołębiowski, M., Maliński, E., Nawrot, J., Szafranek, J., Stepnowski, P. 2007. Identification of the cuticular lipid composition of the western flower thrips *Frankliniella occidentalis*. *Comparative Biochemistry and Physiology*, Part B **147**: 288-292.

Groves, R. L., Sorenson, C. E., Walgenbach, J. F., Kennedy, G. G. 2001. Effects of imidacloprid on transmission of tomato spotted wilt tospovirus to pepper, tomato and tobacco by *Frankliniella fusca* Hinds (Thysanoptera: Thripidae). *Crop Protection*, **20:** 439-445.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**: 95-98.

Hiromori, H. and Nishigaki, J. 2001. Factor analysis of synergistic effect between the entomopathogenic fungus *Metarhizium anisopliae* and synthetic insecticides. *Applied Entomology and Zoology*, **36**: 231-236.

Howard, A. F. V., N'Guessan, R., Koenraadt, C. J. M., Asidi, A., Farenhorst, M., Akogbéto, M., Thomas, M. B., Knols, B. G. J., Takken, W. 2010. The entomopathogenic fungus *Beauveria bassiana* reduces instantaneous blood feeding in wild multi-insecticide-resistant *Culex quinquefasciatus* mosquitoes in Benin, West Africa. *Parasites and Vectors*, 3: 87.

Hu, G. 2005. Using the fungus *Metarhizium anisopliae* as a model system to study the role of gene duplication, divergence and expression in adapting to pathogenicity, Vol. Ph.D: Department of Entomology (ed. University of Maryland, p. 103.

Inglis, D. G., Goettel, S. M., Butt, M. T., Strasser, H. 2001. Use of Hyphomycetes fungi for managing insect pests: Fungi as Biocontrol Agents (ed. by TM Butt, C Jackson & N Magan) CABI Publishing, Wallingford, pp. 23-27.

Inglis, G. D., Ivie, T. J., Duke, G. M. Goettel, M. S. 2000. Influence of Rain and Conidial Formulation on Persistence of *Beauveria bassiana* on Potato Leaves and Colorado Potato Beetle Larvae. *Biological Control*, **18**: 55–64.

Inoue, T., Murai, T., Natsuaki, T. 2010. An effective system for detecting Iris yellow spot virus transmission by *Thrips tabaci*. *Plant Pathology*, **59**: 422-428.

Irigaray, F. J., Marco-Manceb, V., Ignacio, P. 2003. The entomopathogenic fungus *Beauveria bassiana* and its compatibility with triflumuron: effects on the two spotted spider mite *Tetranychus urticae*. *Biological Control*, **26**: 168–173.

Jacobson, R. J., Chandler, D., Fenlon, J., Russell, K. M. 2001a. Compatibility of *Beauveria bassiana* (Balsamo) Vuillemin with *Amblyseius cucumeris* Oudemans (Acarina: Phytoseiidae) to control *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) on cucumber plants. *Biocontrol Science and Technology*, **11**: 391-400.

Jacobson, R. J., Croft, P. J., Fenlon, J. 2001b. Suppressing establishment of *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) in cucumber crops by prophylactic release of *Amblyseius cucumeris* Oudemans (Acarina: Phytoseiidae). *Biocontrol Science and Technology*, **11**: 27-34.

Jaronski, S. T. 2010. Ecological factors in the inundative use of fungal entomopathogens. *Biocontrol*, **55**: 159-185.

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

Jensen, E. S. 2004. Insecticide resistance in the western flower thrips, *Frankliniella occidentalis*. *Journal of Integrated Pest Management Reviews*, **5**: 131-146.

Jenser, G., Szénási, Á., Almási, A., Gáborjányi, R. 2001. The vector capability of *Thrips tabaci*: Thrips and Tospoviruses: Proceedings of the 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound), Reggio Calabria, Italy.

Jones, D. R. 2005. Plant viruses transmitted by thrips. *European Journal of Plant Pathology*, 113: 119-157.

Kang, S. C., Park, S., Lee, D. G. 1999. Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. *Journal of Invertebrate Pathology*, **73**: 276-281.

Khan, M. A., Khaliq, A., Subhani, M. N., Saleem, M. W. 2008. Incidence and development of *Thrips tabaci* and *Tetranychus urticae* on field grown Cotton. *International Journal of Agriculture and Biology*, **10**: 232-234.

Kirk, W. D. and Hamilton, J. G. C. 2004. Evidence for a male-produced sex pheromone in the western flower thrips *Frankliniella occidentalis*. *Journal of Chemical Ecology*, **30**: 167-174.

Kirk, W. D. J. and Terry, L. I. 2003. The spread of the western flower thrips *Frankliniella occidentalis* (Pergande). *Agricultural and Forest Entomology*, **5:** 301-310.

Koschier, E. H. 2006. Plant allelochemicals in thrips control strategies: Naturally Occurring Bioactive Compounds (ed. by Rai & Carpinella) Elsevier, pp. 221-249

Koschier, E. H. 2008. Essential oil compounds for thrips control. *Natural Product Communications*, **3**: 1171-1182.

Kritzman, A., Raccah, B., Gera, A. 2001. Transmission of Iris Yellow Spot Tospovirus: Thrips and Tospoviruses: Proceedings of the 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound), Reggio Calabria, Italy.

Kuslitzky, W. 2003. New variant: Annotated list of hymenopterous parasitoids of thrips in Israel, Vol. 31: Abstracts 20th Conference of the Entomological Society of Israel (ed. Phytoparasitica.

Lacey, L. 1997. Manual of Techniques Insects Pathology. New York, USA.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. (2001) Clustal W and Clustal X version 2.0. *Bioinformatics*, **23**: 2947-2948.

Leland, J. and Behle, R. W. 2004. Formulation of the entomopathogenic fungus, *Beauveria bassiana*, with resistance to UV degradation for control of tarnished plant bug, *Lygus lineolaris*: Beltwide Cotton Conferences, San Antonio.

Lewis, T. 1997a. Chemical control: Thrips as Crop Pests (ed. by T Lewis) CAB Intl., Wallingford, UK, pp. 567-593.

Lewis, T. 1997b. Thrips as crop pests CAB Wallingford, Oxon, UK.

Li, D-C. 2006. Review of fungal chitinases. *Mycopathologia*, **161**: 345-360.

Li, H., Greene, L. H. 2010. Sequence and structural analysis of the chitinase insertion domain reveals two conserved motifs involved in chitin-binding. *PLoS One*, 5.

Loomans, A. J. M. 2006. Exploration for hymenopterous parasitoids of thrips. *Bulletin of Insectology* 59: 69-83.

Loomans, A. J. M. and Murai, T. 1997. Culturing thrips and parasitoids: Thrips as crop pests (ed. by T Lewis) CAB International, Oxon, UK, pp. 477-503.

Lu, P. M. and Lee, H. S. 1985. The life history and seasonal occurrence of onion thrips (*Thrips tabaci* Lindeman). *Journal of Agricultural Research of China*, **36**: 118-124.

Lublinkhof, J. and Foster, D. E. 1977. Development and reproductive capacity of *Frankliniella occidentalis* (Thysanoptera: Thripidae) reared at three temperatures. *Journal of the Kansas Entomological Society*, 50: 313-316.

Ludwig, S. and Oetting, R. 2001. Effect of spinosad on *Orius insidiosus* (Hemiptera: Anthocoridae) when used for *Frankliniella occidentalis* (Thysanoptera: Thripidae) control on greenhouse pot chrysanthemums. *Florida Entomologist*, **84:** 311-313.

Ludwig, S. W. and Oetting, R. D. 2002. Efficacy of *Beauveria bassiana* plus insect attractants for enhanced control of *Frankliniella occidentalis* (Thysanoptera: Thripidae). Florida Entomologist **85**: 270-272.

Maniania, N. K. 1998. A device for infecting adult tsetse flies, *Glossina* spp., with an entomopathogenic fungus in the field. *Biological Control*, **11**: 248-254.

Maniania, N. K. 2002. A low-cost contamination device for infecting adult tsetse flies, *Glossina* spp., with the entomopathogenic fungus *Metarhizium anisopliae* in the field. *Biocontrol Science Technology*, **12**: 59-66.

Maniania, N. K., Bugeme, D. M., Wekesa, V. W., Delalibera, I. J., Knapp, M. 2008. Role of entomopathogenic fungi in the control of *Tetranychus evansi* and *Tetranychus urticae* (Acari: Tetranychidae), pests of horticultural crops. *Expermental Applied Acarology*, 46: 259-274.

Maniania, N. K., Ekesi, S., Lohr, B., Mwangi, F. 2002. Prospect for biological control of the western flower thrips, *Frankliniella occidentalis*, with the entomopathogenic fungus, *Metarhizium anisopliae* on Chrysanthemum. *Mycopathologica*, **155**: 229-235.

Maniania, N. K., Ekesi, S., Odulaja, A., Okech, M. A., Nadel, D. J. 2006. Prospects of a fungus-contamination device for the control of tsetse fly *Glossina fuscipes fuscipes*. *Biocontrol Science and Technology*, **16**: 129-139.

Maniania, N. K., Sithanantham, S., Ekesi, S., Onu, I., Ampong-Nyarko, K., Baumgärtner, J., Löhr, B., Matoka, C. M. 2003. A field trial of the entomopathogenous fungus *Metarhizium anisopliae* for control of onion thrips, *Thrips tabaci*. *Crop Protection*, **22**: 553-559.

Mburu, D. M., Ndung'u, M. W., Maniania, N. K., Hassanali, A. 2011. Comparison of volatile blends and gene sequences of two isolates of *Metarhizium anisopliae* of different virulence and repellency toward the termite *Macrotermes michaelseni*. *The Journal of Experimental Biology*, **214**: 956-962.

Mburu, D. M., Ochola, L., Maniania, N. K., Njagi, P. G. N., Gitonga, L. M., Ndung'u, M. W., Wanjoya, A. K., Hassanali, A. 2009. Relationship between virulence and repellency of entomopathogenic isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the termite *Macrotermes michaelseni*. *Journal of Insect Physiology*, **55**: 774-780.

Migiro, L. N., Maniania, N. K., Chabi-Olaye, A., Vandenberg, J. 2010. Pathogenicity of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) isolates to the adult pea leafminer (Diptera: Agromyzidae) and prospects of an autoinoculation device for infection in the field. *Environmental Entomology*, **39**: 468-475.

Migiro, L. N., Maniania, N. K., Chabi-Olaye, A., Wanjoya, A., Vandenberg, J. 2011. Effect of infection by *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) on the feeding and oviposition of the pea leafminer *Liriomyza huidobrensis* (Diptera: Agromyzidae) on different host plants. *Biological Control*, **56**: 179-183.

Mnyone, L. L., Kirby, M. J., Mpingwa, M. W., Lwetoijera, D. W., Knols, B. G. J., Takken W., Koenraadt, C. J. M., Russell, T. L. 2011. Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood-feeding status. *Parasitology Research*, **108**: 317-322.

Mohan, M. C., Reddy, N. P., Devi, U. K., Kongara, R., Sharma HC (2007) Growth and insect assays of *Beauveria bassiana* with neem to test their compatibility and synergism. *Biocontrol Science and Technology*, **17**: 1059-1069.

Moore, D., Reed, M., Le Patourel, G., Abraham, Y. J., Prior, C. 1992. Reduction in feeding by the desert locust, *Schistocerca gregaria*, after infection with *Metarhizium flavoviride*. *Journal of Invertebrate Pathology*, **60**: 304-307.

Moreira, S., Castanheira P., Casal, M., Faro, C., Gama, M. 2010. Expression of the functional carbohydrate-binding module (CBM) of human laforin. *Protein Expression and Purification*, **74**: 169-174.

Morishita, M. 2008. Pyrethroid-resistant onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), infesting persimmon fruit. *Applied Entomology and Zoology*, **43**: 25-31.

Moritz, G. 1997. Structure, growth and development: Thrips as crop pest (ed. by T Lewis) CAB International, Wallingford, UK, pp. 1-63.

Moritz, G., Morris, D. C., Mound, L. A. 2001. An interactive identification and information system: Thrips ID pest of the world: CSIRO Publishing, Collingwood Australia.

Morsello, S. C. 2007. The role of temperature and precipitation on thrips populations in relation to the epidemiology of Tomato Spotted Wilt Virus, Vol. Ph.D: Entomology (ed. North Carolina State University, Raleigh, North Carolina, p. 126.

Mound, L. A. 2001. So many thrips – so few tospoviruses?: Thrips and Tospoviruses: Proceedings of the 7th International Symposium on Thysanoptera (ed. by R Marullo & L Mound), Reggio Calabria, Italy.

Murai, T., Imai, T., Maekawa, M. 2000. Methyl anthranilate as an attractant for two thrips species and the thrips parasitoid *Ceranisus menes*. *Journal of Chemical Ecology*, **26**: 2557-2565.

Musumeci, S. and Paoletti, G. M. 2009. Binomium chitin-chitinase: Recent issues. Nova Science Publishers, New York.

Muvea, A. M., Kutima, H. L., Waiganjo, M. M., de Kogel, W. J., Teulon, D. A. J., Lagat, Z. O., Subramanian, S. 2010. The potential of coloured sticky traps with LUREM TR attractants for determining population dynamics of pest thrips on tomatoes in Kenya. In: 10th Workshop on Sustainable Horticultural Production in the Tropics Dec 8th - 11th, 2010, JKUAT, Kenya

Nakahara, S. and Monteiro, R. C. 1999. *Frankliniella zucchini* (Thysanoptera: Thripidae), a new species and vector of Tospovirus in Brazil, Vol. 101: *Entomological Society of Washington*, p 290-294.

Nana, P., Maniania, N. K., Maranga, R. O., Kutima, H. L., Boga, H. I., Nchu, F., Eloff, J. N. 2010. Attraction response of adult *Rhipicephalus appendiculatus* and *Rhipicephalus pulchellus* (Acari: Ixodidae) ticks to extracts from *Calpurnia aurea* (Fabaceae). *Veterinary Parasitology*, **174**: 124-130.

Navon, A. and Aschers, K. R. S. 2000. Bioassays of entomopathogenic Microbes. CABI Internatinal Press, Wallinford, UK.

Nchu, F., Maniania, N. K., Toure, A., Hassanali, A., Eloff, J. N. 2009. The use of a semiochemical bait to enhance exposure of *Amblyomma variegatum* (Acari: Ixodidae) to *Metarhizium anisopliae* (Ascomycota: hypocreales). *Veterinary Parasitology*, 160: 279-284.

Nderitu, J. H., Kasina, J. M., Nyamasyo, G. N. 2008. Management of thrips (Thysanoptera: Thripidae) on French bean (Fabaceae) in Kenya: Economics of insecticide applications. *Journal of Entomology*, **5**: 148-155.

Nderitu, J. H., Wambua, E. M., Olubayo, F., Kasina, J. M., Waturu, C. N. 2007. Management of thrips (Thysanoptera: Thripidae) infestation on French Beans (*Phaseolus vulgaris* L.) in Kenya by combination of Insecticides and varietal resistance. *Journal of Entomology*, **4**: 469-473.

Neves, P. M. O., Hirose, J. E., Tchujo, P. T., Moin, J. A. 2001. Compatibility of entomopathogenic fungi with neonicotinoid insecticides. *Neotropical Entomology*, 30: 63–68.

Ngakou, A., Tamo,` M., Parh, I. A., Nwaga, D., Ntonifor, N. N., Korie, S., Nebane, C. L. N. 2008. Management of cowpea flower thrips, *Megalurothrips sjostedti* (Thysanoptera, Thripidae), in Cameroon. *Crop Protection*, **27**: 481-488.

Ondiaka, S., Maniania, N. K., Nyamasyo, G. H. N., Nderitu, J. H. 2008. Virulence of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to sweet potato weevil *Cylas puncticollis* and effects on fecundity and egg viability. *Annals of Applied Biology*, **153**: 41–48.

Poprawski, T. J., Greenberg, S. M., Ciomperlik, M. A. 2000. Effect of host plant on *Beauveria bassiana* and *Paecilomyces fumosoroseus*-induced mortality of *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). *Environmental Entomology*, 29.

Quesada-Moraga, E., Martin-Carballo, I., Garrido-Jurado, I., Santiago-Álvarez, C. 2008. Horizontal transmission of *Metarhizium anisopliae* among laboratory populations of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Biological Control 47: 115-124.

Rachappa, V., Lingappa, S., Patil, K. 2007. Effect of agrochemicals on growth and sporulation of *Metarhizium anisopliae* (Metschnikoff) Sorokin. *Karnataka Journal of Agricultural Science* **20**: 410-413.

Rai, M. and Kovics, G. 2010. Progress in Mycology. Springer Scientific Publishers, New Pali Road, India.

Rhainds, M., Cloutier, C., Shipp, L., Boudreault, S., Daigle, G., Brodeur, J. 2007. Temperature-mediated relationship between western flower thrips (Thysanoptera: Thripidae) and Chrysanthemum. *Environmental Entomology*, **36**: 475-483.

Saxena, R. C. and Kidiavai, E. L. 1997. Neem seed extract spray applications as low-cost inputs for management of the flower thrips in the cowpea crop. *Phytoparasitica*, **25**: 99-110.

Scholte, E-J., Ng'habi, K., Kihonda, J., Takken, W., Paaijmans, K., Abdulla, S., Killeen, G. F., Knols, B. G. J. 2005. An entomopathogenic fungus for control of adult african malaria mosquitoes. *Science*, **308**: 1641-1642.

Screen, S. E. and St-Leger R. J. 2000. Cloning, Expression, and Substrate Specificity of a Fungal Chymotrypsin: evidence for lateral gene transfer from an actinomycete Bacterium. *The Journal of Biological Chemistry*, 275: 6689-6694.

Seidl, V., Gamauf, C., Druzhinina, I. S., Seiboth, B., Hart, L., Kubicek, C. P. 2008. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. *BMC Genomics*, **9**.

Seidl, V., Song, L., Lindquist, E., Gruber. S., Koptchinskiy, A., Zeilinger, S., Schmoll, M., Martínez, P., Sun, J., Grigoriev, I., Herrera-Estrella, A., Baker, S. E., Kubicek, C. P. 2009. Transcriptomic response of the mycoparasitic fungus *Trichoderma atroviride* to the presence of a fungal prey. *BMC Genomics*, **10**.

Seyoum E., Moore, D., Charnley, A. K. 1994. Reduction in flight activity and food consumption by the desert locust *Schistocerca gregaria* Forskal (Orth.: Cyrtacanthacrinae), after infection with *Metarhizium flavoviride*. *Journal of Applied Entomology* 118: 310-315.

Shi, W-B., Jiang, Y., Feng, M-G. 2005. Compatibility of ten acaricides with *Beauveria bassiana* and enhancement of fungal infection to *Tetranychus cinnabarinus* (Acari: Tetranychidae) eggs by sublethal application rates of pyridaben. *Applied Entomology and Zoology*, **40**: 659–666.

Soldatenkov, A. T. and Kolyadina, N. M. 2001. Chemistry of indolopyridines with a bridgehead heteroatom. *Chemistry of Heterocyclic Compounds*, **37**: 1059-1091.

Soper, R. S. and Ward, M. G. 1981. Beltsville Symposia in Agricultural Research. *Biological Control in Crop Production*, **5**: 161-180.

Steiner, M. Y., Goodwin, S., Wellham, T. M., Barchia, I. M., Spohr, L. J. 2003. Biological studies of the Australian predatory mite *Typhlodromips montdorensis* (Schicha) (Acari: Phytoseiidae), a potential biocontrol agent for western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). *Australian Journal of Entomology*, **42**: 124.

St-Leger, R. J., Joshi, L., Bidokha, M. J., Rizza, N. W., Roberts, D. W. 1996. Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. *Applied and Environmental and Microbiology*, **62**: 907-912.

Stolz, I., Nagel, P., Lomer C., Peveling, R. 2002. Susceptibility of the Hymenopteran *Parasitoids Apoanagyrus* (= *Epidinocarsis*) *lopezi* (Encyrtidae) and *Phanerotoma sp*. (Braconidae) to the Entomopathogenic Fungus *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes). *Biocontrol Science and Technology*, **12**: 349-360.

Subramanian, S., Maniania, N. K., Nyambo, B. 2009. Progress Report: Integrated Control of Thrips in Vegetables in Eastern Africa (ed. by S Subramanian) International Centre of Insect Physiology and Ecology (icipe), Nairobi Kenya.

Teulon, D. A. J. and Penman, D. R. 1992. Colour preferences of New Zealand thrips (Terebrantia: Thysanoptera). *New Zealand Entomologist*, **15**: 9-13.

Teulon, D. A. J., Davidson, M. M. , Nielsen, M-C., Perry, N. B. , van Tol R. W. H. M., de Kogel., W-J. 2008. The potential use of lures for thrips biocontrol in greenhouses: practice and theory: Third International Symposium on Biological Control of Arthropods (ed. by PG Mason, DR Gillespie & C Vincent), Christchurch, New Zealand, pp. 301-308.

Teulon, D., Davidson, M., Nielsen, M., Perry, N., Tol, Rv., de Kogel, W-J., 2010. The lure of scent: Allelochemicals for thrips pest management: Plant research international (ed. Vineland Research and Innovation Centre Wageningen.

Till, C. M., Butler, R. C., Horne, P. A., Hives, N., Teulon, D. A. J. 2009. Using LUREM-TR to trap thrips in glasshouse crops in Victoria, Australia. *New Zealand Plant Protection*, **62**: 398.

Trdan, S., Bergant, K., Jenser, G. 2003. Monitoring of western flower thrips (*Frankliniella occidentalis* [Pergande], Thysanoptera) in the vicinity of greenhouses in different climatic conditions in Slovenia. *Agricultura*, **2**: 1-6.

Trdan, S., Valic, N., Zezlina, I., Bergant, K., Znidarcic, D. 2005. Light blue sticky boards for mass trapping of onion thrips, *Thrips tabaci* Lindeman (Thysanoptera: Thripidae), in onion crops: fact or fantasy? *Journal of Plant Diseases and Protection*, **112**: 173-180.

Tsuda, S., Fujisawa, I., Ohnishi, J., Hosokawa, D., Tomaru, K. 1996. Localization of Tomato Spotted Wilt Tospovirus in larvae and pupae of the insect vector *Thrips setosus*. *Phytopathology* **86**: 1199-1203.

Ugine, T A., Wraight, S. P., Sanderson, J. P. 2006. Influences of impatiens pollen and exposure to *Beauveria bassiana* on bionomics of western flower thrips *Frankliniella occidentalis*. Biological Control 37: 186-195.

Ugine, T. A., Wraight, S. P., Sanderson J. P. 2007b. A tritrophic effect of host plant on susceptibility of western flower thrips to the entomopathogenic fungus *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **96**: 162-172.

Ugine, T. A., Wraight, S. P., Sanderson, J. P. 2005. Acquisition of lethal doses of *Beauveria bassiana* conidia by western flower thrips, *Frankliniella occidentalis*, exposed to foliar spray residues of formulated and unformulated conidia. *Journal of Invertebrate Pathology*, 90: 10-23.

Ugine, T. A., Wraight, S. P., Sanderson, J. P. 2007a. Effects of manipulating spray-application parameters on efficacy of the entomopathogenic fungus *Beauveria bassiana* against western flower thrips, *Frankliniella occidentalis*, infesting greenhouse impatiens crops. *Biocontrol Science and Technology*, **17**: 193- 219.

Ullman, D. E., Meideros, R., Campbell, L. R., Whitfield, A. E., Sherwood, J. L., German, T. L. 2002. Thrips as vectors of tospoviruses. *Advances in Botanical Research*, 36: 113-140.

Ullman, D. E., Westcot, D. M., Chenault, K. D., Sherwood, J. L., German, T. L., Bandla, M. D., Cantone, F. A., Duer, H. L. 1995. Compartmentalization, intracellular transport, and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. *Phytopathology*, **85**: 44-54.

Van-Tol, R. W. H. M., James, D. E., De Kogel, W. J., Teulon, D. A. J. 2007. Plant odours with potential for a push-pull strategy to control the onion thrips, *Thrips tabaci*. *Entomologia Experimentalis et Applicata*, **122**: 69-76.

Vega F. E., Dowd, P. F., Lacey, L. A., Pell, J. K., Jackson, D. M., Klein, M. G. 2007. Dissemination of beneficial microbial agents by insects: Field manual of techniques in invertebrate pathology (ed. by **LA Lacey and HK Kaya**) Springer, Dordrecht,The Netherlands, pp. 127-146.

Venzon, M., Janssen., A., Sabelis, M. W. 2002. Prey preference and reproductive success of the generalist predator *Orius laevigatus*. *Oikos*, **97**: 116.

Vestergaard, S. 1995. Pathogenicity of the Hyphomycete Fungi *Verticillium lecanii* and *Metarhizium anisopliae* to the Western Flower Thrips, *Frankliniella occidentalis*. Biocontrol Science and Technology 5: 185-192.

Waiganjo, M. M., Sithanantham S., Mueke, J. M., Gitonga, L. M. 2006. Onion seedling protection against *Thrips tabaci* Lindman and its comparative economic benefit over pesticides spray: 10th KARI Biennial Scientific Conference (ed. by KAR Institute), Kenya.

Wei, W. and Mei, Z-H. 2009. 2-(1H-Benzotriazol-1-yl)-1-(3-methoxybenzoyl) ethyl isonicotinate: Acta Crystallographica Section E (ed. College of Life Science and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing, Jiangsu.

White, T. J., Bruns, T., Lee, S., Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, New York.

Whitfield, A. E., Ullman, D. E. German, T. L. 2005. Tospovirus-Thrips interactions. *Annual Review in Phytopathology*, **43**: 59-89.

Zen S., Okuda M., Fuji, S., Iwanami, T. 2008. The seasonal occurrence of viruliferous *Thrips tabaci* and the incidence of iris yellow spot virus disease on Lisianthus. *Journal of Plant Pathology*, **90**: 511-515.

Zhang, S., Wu, L., Xu, X., Hua, B. 2006. Predation of *Orius minutus* on *Odontothrips loti*. Ying Yong Sheng Tai Xue Bao: 1259–1263.