Entomopathogenic fungi: a component of leafminer management

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A thesis submitted in fulfillment of the requirements for the award of the degree Doctor of Philosophy in Environmental Sciences, North-West University (Potchefstroom campus)

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May 2010

DEDICATION

To my parents Elijah and Agnes Migiro and the entire family for constant love and support

To my late grandfather Pius Migiro “moong’era bokong’u”

ACKNOWLEDGEMENTS

I am grateful to the International Centre of Insect Physiology and Ecology (ICIPE) and North-West University for providing me the necessary support and the opportunity to study in the two institutions. The German Academic Exchange Services (DAAD), the German Federal Ministry for Economic Cooperation and Development (BMZ), the Arthropod Pathology Unit, the Leafminer Project and Capacity and Institutional Building are greatly acknowledged for the financial support.

This work would not have been possible without the immense support and superb supervision from my advisors Prof. Johnnie van den Berg of North-West University, Dr. Nguya Maniania and Dr. Chabi Olaye of ICIPE. My heartfelt appreciation for your kind advice, mentorship, patience and keenness to detail and the little push which I surely needed. I am extremely lucky to have had a chance to learn from you.

I am grateful for the unwavering support of the staff of the Arthropod Pathology Unit and Leafminer project, it was a pleasant and fruitful experience working with you all**.** Specifically, I am highly indepted to Elizabeth Ouna for her technical support in laboratory techniques and to Sospeter Wafula and Shem Ondiaka for their assistance in the laboratory. I am also grateful to Dr. Haas Fabian (Biosystematics, unit) for the leafminer photographs and Mr. Mwanga Komeri (Science Press) for drawing the autoinoculation device.

I want to thank all the people who contributed to this thesis mainly through their moral support and invaluable friendship. To the following friends and colleagues, Nigat Bekele, Katharina Merkel, Mary Nelima, Susan Sande, Fikira Kimbokota, Ylva Pherson, Edda Nangole, Bonaventure Aman, Yusuf Abdullahi, Robert Musundire, Benjamin Muli, David Mburu, Duna Mailafyia, Meshack Obonyo, Saliou Niassy and Obadiah Mucheru, I am grateful.

Finally, to all my family members, your love, encouragement, understanding and support has always being unquestionable throughout my life. Thank you and may God richly reward you.

ABSTRACT

The invasive leafminers *Liriomyza sativae* (Blanchard), *Liriomyza trifolii* (Burgess) and *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) are major pests of many vegetable and ornamental crops worldwide. In Kenya, production of horticultural crops is also severely constrained by infestation of *Liriomyza* leafmining flies (LMF), especially the invasive *L. huidobrensis*. Being quarantine pests, their presence in export produce can lead to rejections, resulting in loss of export markets and consequently loss of revenue to many smallholder families that are involved in export crop production. These constraints to trade represent the newest and potentially most challenging limitation to the future development of the horticultural sector in Kenya. Farmers increasingly use mixtures of chemical insecticides and spray more frequently in response to damage by key pests such as LMF. As a result, environmental contamination, health risks, pesticide residues and production costs are increasing. Increased use of pesticides also constrains the impact of the pest's natural enemies and LMF have already developed resistance to several insecticides. The development of insecticide resistance has stimulated an increased interest in the search for non-chemical control measures such as the use of parasitoids, resistant plant varieties, entomopathogenic nematodes and entomopathogenic fungi as alternatives to chemicals. The current study is part of a larger research project on self-sustaining pest management strategies for *Liriomyza* species in Kenyan horticultural systems. The objective of this study was to investigate the potential of entomopathogenic fungi *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) for the control of *L. sativae*, *L. trifolii* and *L. huidobrensis.* The pathogenicity of 17 isolates of *M. anisopliae* and three isolates of *B. bassiana* to *L. huidobrensis* was evaluated in the laboratory. All the isolates were pathogenic to the leafminer causing mortality of 40 to 100% at five days post-exposure. The lethal time for 50% mortality (LT_{50}) ranged from 2.6 to 5.4 days while the LT_{90} values varied between 3.2 and 9.1 days depending on the isolate. Ten isolates of *M. anisopliae* (ICIPE 315, 69, 78, 07, 60, 62, 84, 20, 387,

18) and three of *B. bassiana* (ICIPE 273, 603, 279) were found to be highly virulent. An autoinoculation device for field application of fungus was developed and tested in cage experiments using only one of the virulent isolates, *M. anisopliae* ICIPE 20. Mortality of up to 100% was observed in flies captured from fungus-treated cages held under laboratory conditions. This indicates that leafminer flies were attracted to the device and were able to pick up a lethal dose of inoculum (4.1×10^5 to 4.0×10^6 conidia per fly), resulting in high adult mortality. One day after the inoculation, adults picked-up an average of $4.1 \pm 0.7 \times 10^5$ conidia and $39.6 \pm 4.0 \times 10^5$ conidia five days post-inoculation. Depending on the sampling date, the LT_{50} varied between 1.8 and 3.4 days. The effect of fungal infection by *M. anisopliae* ICIPE 20 on feeding and oviposition of adult *L. huidobrensis* was examined on three host plants, i.e. faba bean, *Vicia faba* L., snow pea, *Pisum sativum* L. (cv. Oregon II) and French bean, *Phaseolus vulgaris* L. (cv. Samantha) (Fabales: Fabaceae), in the laboratory. Infection by *M. anisopliae* significantly reduced feeding and oviposition by *L. huidobrensis*. However, reductions in punctures and eggs generally occurred after 72 h post-inoculation. The host plant did not have any effect on the feeding but had an influence on the egg laying, with faba bean harboring a greater number of eggs in both the control and the *M. anisopliae* treatments. Insects reared on faba bean were less susceptible to fungal infection than those reared on French bean and snow pea. The effect of constant temperatures on the virulence of five isolates of *M. anisopliae* against the three species of leafminers, *L. huidobrensis, L. sativae* and *L. trifolii* was studied in the laboratory. Insect mortality varied with temperature, fungal isolate and leafminer species. Results showed that fungal isolates were more virulent at 25°C and 28°C than at 15°C and 20°C and that, lethal time to 50% mortality (LT_{50}) values decreased with increasing temperature. In another set of experiments, the effect of two host plants, French bean and faba bean on the susceptibility of the three leafminer species to one of the virulent *M. anisopliae* isolates (ICIPE 20) was also investigated. Leafminer mortality was affected by both the concentrations used (1×10^5, 10^6, 10^7 conidia ml^{-1}) and the host plant. Insects reared on faba bean plants seemed to take

longer to succumb to infection. *Liriomyza huidobrensis* reared on faba bean required higher conidial concentrations to kill compared to those reared on French bean. However, for both *L. sativae* and *L. trifolii*, host plant had no effect on concentration. The effect of the entomopathogenic fungus *M. anisopliae* isolate, ICIPE 20 on the leafminer parasitoid *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) was investigated in the laboratory. Results showed that *M. anisopliae* was pathogenic to *D. isaea* adults causing up to 76% mortality at six days after inoculation and affected the emergence of the parasitoids. Results of this study demonstrate the potential of entomopathogenic fungi *M. anisopliae* and *B. bassiana* for the management *of L. huidobrensis, L. trifolii* and *L. sativae*.

UITTREKSEL

Die indringer-bladmynerspesies *Liriomyza sativae* (Blanchard), *Liriomyza trifolii* (Burgess) en *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) word wêreldwyd beskou as belangrike plae van groente en ornamentele gewasse. Ook in Kenia word produksie van tuinbougewasse ernstig benadeel deur infestasies van *Liriomyza* bladmyners, veral die indringerspesie *L. huidobrensis*. Aangesien bladmyners as kwarantynplae beskou word kan hulle aanwesigheid in uitvoerprodukte lei tot afkeur van produkte met gevolglike verlies van uitvoermarkte en inkomste vir kleinboerfamilies wat betrokke is by produksie vir die uitvoermark. Hierdie is uitdagende beperkinge vir die toekomstige ontwikkeling van die tuinbousektor in Kenia. Boere maak toenemend gebruik van insekdodermengsels en bespuit meer gereeld as gevolg van skade deur sleutelplae soos bladmyners. As gevolg hiervan is daar ’n toename in omgewingsbesoedeling, gesondheidsrisiko’s, plaagdoder-residue asook ’n toename in produksiekoste. Die gebruik van insekdoders beperk ook die impak van die natuurlike vyande van plae. Bladmyners het ook reeds bestandheid ontwikkel teen verskeie tipes insekdoders. Hierdie ontwikkeling van insekdoderweerstand het gelei tot verdere ondersoeke na nie-chemiese beheermetodes soos bv. die gebruik van parasitoïde, weerstandbiedende plantvariëteite, entomopatogeniese nematode en fungi as alternatief vir chemiese beheer. Die huidige studie vorm deel van ’n groter projek oor volhoubare plaagbestuur van *Liriomyza* spesies in tuinboustelsels in Kenia. Die doel van hierdie studie was om die potensiaal van die entomopatogeniese fungi *Metarhizium anisopliae* (Metchnikoff) Sorokin en *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) te ondersoek vir die beheer van *L. sativae*, *L. trifolii* en *L. huidobrensis.* Die patogenisiteit van 17 isolate van *M. anisopliae* en 3 isolate van *B. bassiana* vir *L. huidobrensis* is in laboratoriumstudies ge-evalueer. Alle isolate was patogenies vir bladmyners en het mortaliteit van tussen 40 en 100% tot gevolg gehad, vyf dae na blootstelling. Die letale tydsperiode tot 50% mortaliteit (LT_{50}) het varieer vanaf 2.6 tot 5.4 dae terwyl LT_{90}-waardes tussen 3.2 en 9.1 dae was, afhangend van die isolaat. Tien

isolate van *M. anisopliae* (ICIPE 315, 69, 78, 07, 60, 62, 84, 20, 387, 18) en drie van *B. bassiana* (ICIPE 273, 603, 279) is bevind om hoogs virulent te wees. 'n Outo-inokuleringstoestel vir veldtoediening van fungus is ontwikkel en getoets onder semi-veldtoestande met een van die virulente isolate van *M. anisopliae* (ICIPE 20). Mortaliteit van tot 100% is waargeneem van vlieë wat in fungus-behandelde hokke gevang is en dan onder laboratoriumtoestande aangehou is. Hierdie resultaat dui aan dat bladmyners na die inokuleringstoestel toe aangelok is en dat hulle 'n letale dosis (4.1×10^5 tot 4.0×10^6 konidia per vlieg) van die inokulum opgeneem het. Een dag na inokulasie is $4.1 \pm 0.7 \times 10^5$ konidia per vlieg opgeneem terwyl die getal op vyf dae na inokulasie, $39.6 \pm 4.0 \times 10^5$ per vlieg was. Afhangend van die monsternemingsdatum het die LT_{50} varieer tussen 1.8 en 3.4 dae. Die effek van fungusinfeksie deur *M. anisopliae* ICIPE 20 op voeding en eierlegging van volwasse *L. huidobrensis* is in 'n laboratoriumstudie op drie gasheerplantspesies ondersoek nl. Faba-boontjies, *Vicia faba* L., ertjies, *Pisum sativum* L. (cv. Oregon II) en Franse boontjies, *Phaseolus vulgaris* L. (cv. Samantha) (Fabales: Fabaceae). Infeksie met *M. anisopliae* het gelei tot betekenisvolle afname in voeding en eierlegging deur *L. huidobrensis.* 'n Afname in voedingsgaatjies asook eiergetalle is egter eers 72 uur na inokulasie waargeneem. Gasheerplantspesie het nie 'n invloed gehad op voeding nie, maar wel op eierlegging, met Faba-bone wat 'n groter aantal eiers op beide die *M. anisopliae*-behandelde sowel as kontroleplante getoon het. Insekte wat op Faba-bone geteel is was minder vatbaar vir fungusinfeksie as die wat op Franse bone of ertjies geteel is. Die effek van konstante temperature op virulensie van vyf isolate *M. anisopliae* teen drie spesies bladmyners, *L. huidobrensis, L. sativae* en *L. trifolii* is onder laboratoriumtoestande bestudeer. Resultate het getoon dat insekmortaliteit varieer met temperatuur, fungus-isolaat en bladmynerspesie. Fungus-isolate was meer virulent by 25°C en 28°C as 15°C en 20°C. Die letale tyd tot 50% mortaliteit (LT_{50}) het afgeneem met 'n toename in temperatuur. In 'n ander stel eksperimente is die effek van twee gasheerplante nl. Faba bone en Franse boontjies op die vatbaarheid van drie bladmynerspesies vir die virulente isolaat van *M. anisopliae* (isolate ICIPE 20) ge-evalueer. Bladmynermortaliteit is

beïnvloed by beide konsentrasies wat gebruik is (1 x 10^5, 10^6, 10^7 konidia ml^{-1}) asook deur gasheerplant. Dit wil voorkom asof bladmynervlieë wat op Faba-bone voed langer neem asook hoër konsentrasies konidia nodig het om te vrek as vlieë wat op Franse bone voed. Die effek van die entomopatogeniese fungus *M. anisopliae* isolaat, ICIPE 20 op die bladmyner-parasitoïd *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) is onder laboratoriumtoestande bestudeer en daar is gevind dat dit patogenies was vir *D. isaea* volwassenes. Swaminfeksie het gelei tot mortaliteit van tot 76% ses dae na inokulasie en het ook die proses beïnvloed waar volwasse parasitoïde uit vliegpapies uitkom. Resultate van hierdie studie demonstreer die potensiaal van die entomopatogeniese fungi *M. anisopliae* en *B. bassiana* vir die bestuur van *L. huidobrensis, L. trifolii* en *L. sativae*.

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CHAPTER ONE

1. General introduction and literature review

1.1 General introduction

The Family Agromyzidae (Diptera) contains some of the world's most destructive pests of vegetable and floricultural crops (Spencer, 1973; Parrella 1982; Minkenberg and Van Lenteren, 1986). *Liriomyza* species (Diptera: Agromyzidae) are exclusively plant feeders and are virtually ubiquitous (Spencer, 1973, 1989). Three major pest species of *Liriomyza* leaf miners: *Liriomyza sativae* (Blanchard), *Liriomyza trifolii* (Burgess) and *Liriomyza huidobrensis* (Blanchard) are a threat to horticultural field crops worldwide (Murphy and LaSalle, 1999; Reitz and Trumble, 2002). These particular species are characterized by their high degree of polyphagy, multivoltine nature, ability to develop insecticide resistance rapidly and the extent to which they have invaded new geographical areas including large parts of the old world. Other polyphagous species include *Liriomyza strigata* (Meigen) and *Liriomyza bryoniae* (Kaltenbach) which occur exclusively in the Palearctic region (Spencer, 1973; Liu *et al*., 2008).

According to Spencer (1973), *L. sativae* is native to the Americas while *L. trifolii* is native to south-eastern North America and *L. huidobrensis* is native to South America. *Liriomyza trifolii* was introduced into Kenya in 1976 through chrysanthemum (*Chrysanthemum* spp.) (Asterales: Asteraceae)) cuttings from Florida USA (Spencer, 1985). *Liriomyza sativae* and *L. huidobrensis* have been reported to be present in Kenya although there are no records on their arrival (ICIPE, unpublished data).

Leafminers damage crops by puncturing the leaf surface to feed on exuding sap and to lay eggs into the leaf tissue (Knodel-Montz *et al*., 1985). When the eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines. Leaf mines and punctures reduce the quality of high value horticultural crops in addition to reducing the photosynthetic ability of the plant (Foster and

Sanchez, 1988; Kox *et al*., 2005). During outbreaks, severe infestations from both adult puncturing and larval-mining can lead to total crop losses (Spencer, 1973; 1990). Economic importance is also due to difficulty in their control. They are listed as regulated pests in the EU Plant Health Directive 2000/29 (EU, 2000) hence in addition to the direct losses, losses also stem from the restriction in trade and loss of export markets.

In Kenya, damage by leafminers has been recorded on various crops. *Liriomyza trifolii* has been recorded on sunflower (*Helianthus annuus* Linnaeus (Asterales: Compositae)) at Hola irrigation scheme, and tomatoes (*Lycopersicon esculentum* Mill) (Solanales: Solanaceae)), melons (*Cucumis melo* Linnaeus) and courgettes (*Cucurbita pepo* Linnaeus) (Cucurbitales: Cucurbitaceae)), okra (*Abelmoschus esculentus* (Linnaeus) Moench) (Malvales: Malvaceae)), onions (*Allium cepa* Linnaeus) (Asparagales: Alliaceae)) and beans (*Phaseolus vulgaris* Linnaeus) (Fabales: Fabaceae)) in Thika (Kabira, 1985), and on tomatoes in the Voi area west of Mombasa (Spencer, 1985). *Liriomyza huidobrensis* has been reported to cause damage on vegetables and other ornamental plants such as passion fruit (*Passiflora edulis* Sims (Malpighiales: Passifloraceae)), snow peas (*Pisum sativum* Linnaeus var. *saccharatum*) (Fabales: Fabaceae)), and gypsophila (*Gypsophila* spp.) (Caryophyllales: Caryophyllaceae)) (KEPHIS, 2005). *Liriomyza sativae* has been recorded on tomato, passion fruit, cucumber (*Cucumis sativus* Linnaeus) (Cucurbitales: Cucurbitaceae)) and cowpea (*Vigna unguiculata* (Linnaeus) Walp) (Fabales: Fabaceae)) (ICIPE, unpublished data).

The management of agromyzid leafminers by both smallholder and large-scale producers worldwide has largely relied on chemical insecticides such as carbamates, organophosphates and pyrethroids (Murphy and La Salle, 1999). However, the indiscriminate and frequent use of these chemicals has resulted in insecticide resistance of flies as well as elimination of their natural enemies (MacDonald, 1991; Weintraub and Horowitz, 1995; Murphy and LaSalle, 1999). Other non-chemical leafminer control methods which include the use of

parasitoids (Minkenberg and Van Lenteren, 1986; Waterhouse and Norris, 1987; Johnson, 1993), trapping by yellow sticky traps (Price *et al*., 1981; Bennett, 1984), resistant plant varieties (CIP, 1993) and use of entomopathogenic nematodes (Williams, 1993; Walters *et al*., 2000) have been attempted with varied levels of success. There have been few attempts to use entomopathogenic fungi against the dipteran leafminer flies (Borisov and Ushchekov, 1997; Bordat *et al.,* 1988); but these studies were limited to the screening of fungal isolates against pupae in the laboratory.

1.2 Literature Review

1.2.1 Classification of leafminers

The leafminers belong to the order Diptera in the family Agromyzidae. At present, there are 25 recognized genera in this family and 75% of the known 1800 species are leafminers (Spencer, 1973). *Liriomyza* is one of the largest agromyzid genera, with over 330 described species (Liu *et al*., 2008), out of which only 12 species are known to occur in Africa (Spencer, 1985). There has been considerable taxonomic confusion in the past with regard to the polyphagous Agromyzidae. This has been particularly true with members of the genus *Liriomyza*, due to their wide, overlapping host ranges and general morphological similarity (Parrella, 1982). Parrella (1982) gives an account of the taxonomic confusion of five economically important *Liriomyza* sp. (*L. huidobrensis, L. sativae, L. trifolii, L. brassicae* (Riley) and *L. trifoliearum* Spencer).

1.2.2 Distribution of leafminers

Liriomyza are widely distributed both in the old and new worlds. They mostly occur in temperate regions and in insignificant numbers in the tropics (Parrella, 1987). The three major pest species described above are all Nearctic and Neotropical in distribution. They are believed to be native to the Pacific region extending from the southern United States to northern South America and have been reported to invade almost all zoogeographical regions, partly due to the

development of the cut flower trade (Waterhouse and Norris, 1987). In Kenya, the genus appears to be poorly represented (Spencer, 1985). *Liriomyza trifolii* has been reported in Kibwezi, Athi River, Nairobi, Naivasha, Ruaraka, Thika, Isiolo and at the Hola irrigation scheme (Spencer, 1985). *Liriomyza huidobrensis* has been reported in Naivasha while *L. sativae* has been reported in Kakuzi, Muhaka and Kibwezi (ICIPE, unpublished data).

1.2.3 Economic importance of leafminers

Of the more than 300 *Liriomyza* species described to date, only 23 have been reported as being economically important with five of these being truly polyphagous (Spencer, 1973). In Peru, potato losses of more than 30%, were reported due to *L. huidobrensis* (Weintraub and Horowitz, 1995). In Vanuatu in the 1980's, *L. sativae* caused losses of up to 70% in tomato crops (Waterhouse and Norris, 1987). In Kenya, chrysanthemums were grown commercially before 1976, but *L. trifolii* was thought to have been introduced in contaminated cuttings from Florida (USA), at a large propagating nursery at Masongaleni. By 1979 the nursery was closed, but the establishment of the pest in local wild hosts, and the dissemination of cuttings from the nursery to other parts of the country as well as abroad, has added *L. trifolii* to the pest spectrum of East Africa. Female flies puncture the leaves of the host plants with their ovipositor, causing wounds which serve as sites for feeding or oviposition. When the eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines (Spencer 1973, Knodel-Montz *et al*., 1985; Ameixa *et al*., 2007). Leaf mines and punctures reduce the photosynthetic ability and the quality of high value horticultural crops (Spencer 1973, 1990, Kox *et al*., 2005). When heavy infestations occur damage may lead to total crop losses (Spencer 1973, 1990).

1.2.4 Host plants

Liriomyza leafminers attack a wide range of vegetable and horticultural crops (Waterhouse and Norris, 1987; Murphy and La Salle, 1999; Belokobylskij *et al.*, 2004) and have also been recorded on several wild hosts (Spencer, 1973, 1985).

Liroimyza sativae damages crops mainly in the family Cucurbitaceae, Leguminosae and Solanaceae. In addition, a further 20 genera in 10 families have been recorded as hosts (Spencer, 1989). Stegmaier (1966) listed 47 plant genera in ten families in which *L. trifolii* has been observed, with 40% of favoured hosts being Compositae and almost 15% in the Leguminosae family. *Liriomyza huidobrensis* has been recorded on at least 14 plant families (Spencer 1990).

1.2.5 Biology

1.2.5.1 Life history

The biology of the three species is broadly similar (Waterhouse and Norris 1987). Their lifecycle comprises of an egg stage, three larval stages, a pupal stage and an adult stage. Mating can occur at any time, but is most frequent in daylight hours and within one day of emergence (Parrella, 1987; Murphy and LaSalle, 1999). A single mating ensures all the eggs are fertilized (Minkenberg and Van Lenteren, 1986). Oviposition begins within a day or so of emergence of the females and peaks after about a week but may continue at a decreased rate for several weeks (Parrella and Bethke, 1984; Murphy and LaSalle, 1999). Parrella (1984) reported that the optimal temperatures for feeding and egg-laying for *L. trifolii* ranged between 21°C and 32°C and reduced at temperatures below 10°C.

1.2.5.2 Eggs

Eggs are laid singly in punctures in the leaf epidermis. There is no preference for upper or lower surfaces. The freshly laid eggs are creamy white and shaped like an elongated oval. The eggs are small and vary in size depending on the size of the species. For instance, those of *L. huidobrensis* measure 0.28 mm x 0.15 mm. Eggs hatch after 2-8 days, depending on temperature (Parrella, 1987).

1.2.5.3 Larvae

The duration of larval development also depends on temperature and probably host plant suitability (Spencer, 1973). The larvae measure about 4 mm in length and 1 mm in breadth. There are three larval stages with each taking about 2-3 days. The larvae are typical maggots of the higher Diptera. During completion of

the third instar, the larvae cut their way out through the epidermis of the leaf, fall to the ground or on to lower leaves and either pupate there, or on the soil surface. Larvae may also burrow a small distance into soil before pupation (Parrella, 1987; Murphy and LaSalle, 1999).

1.2.5.4 Pupae

The pupae are distinctly segmented, oval shaped narrowing at the ends. The duration of the pupal stage varies inversely with temperature and at least 50% of the total development time of a *Liriomyza* individual is spent in this stage. This stage does not feed and development is generally completed in 8 to 11 days (Parrella, 1987). Pupae can remain viable outdoors for several months and are able to withstand freezing temperatures (Charlton and Allen, 1981; Parrella, 1987).

1.2.5.5 Adults

Adult leafminers are small (none exceeding 2.3 mm in length) with black and yellow markings (Waterhouse and Norris, 1987; Murphy and LaSalle, 1999). Adults live for 10-30 days depending on environmental conditions. According to Waterhouse and Norris (1987), *L. sativae* is shiny black on its upper surface and the area between the eyes is yellow whereas the head capsule just behind the eyes is dark. *Liriomyza trifolii* has a more grayish upper thorax with much of the head capsule behind the eye being mostly yellow. *Liriomyza huidobrensis* is a slightly larger leafminer fly with the head capsule being black behind the eye. It is normally darker overall with a more pale-yellow colour than the other species. The life cycle from egg to adult generally takes three weeks (at 30°C) to more than nine weeks (at 14°C) to complete, depending on temperature and host plant species (Charlton and Allen, 1981; Parrella, 1982).

a

b

c

Plate 1.1 Adults of (a) *Liriomyza trifolii*, (b) *L. sativae*, (c) *L. huidobrensis*.

1.2.6 Natural enemies of leafminers

There has been considerable work on natural enemies of leafminers. Waterhouse and Norris (1987) provided a detailed list of the natural enemies of *Liriomyza* spp. and a summary of the results of biological control introductions.

1.2.6.1 Parasitoids

Numerous species of the chalcidoid families Eulophidae (mostly) and Pteromalidae, as well as several genera of Braconidae and eucoiline Figitidae have been recorded as parasitoids of Agromyzidae (Murphy and La Salle, 1999; Noyes, 2009). Waterhouse and Norris (1987) listed more than 40 species of parasitoids from the three *Liriomyza* spp. The parasitoids attack the larval stage of the leafminers and are either ectoparasitic or endoparasitic in habit (Murphy and LaSalle, 1999). When fully developed, some species emerge from within the mine (for example, *Diglyphus* spp. (Eulophidae: Hymenoptera)) and other species from the puparium of the fly after it has fallen to the ground (for example, *Chrysocharis* spp. (Eulophidae: Hymenoptera)) (Minkenberg and Van Lenteren, 1986).

1.2.6.2 Predators

A few predators belonging to five insect orders, i.e. Coleoptera, Hemiptera, Diptera, Dermaptera, Hymenoptera, as well as spiders attack leafminer flies (Cisneros and Mujica, 1998). Most predators are general feeders and prey indiscriminately on several insect pests. Some predatory flies of the families Dolichopodidae and Empididae have been noted attacking leafminers (Cisneros and Mujica, 1998). Soil inhabiting predators such as *Calosoma abbreviatum* Chaudoir and *Pterostichus* sp. (Coleoptera: Carabidae) attack pupae. Foliage inhabiting predators such as *Geocoris punctipes* Say (Hemiptera: Lygaeidae), *Orius insidiosus* Say (Hemiptera: Anthocoridae) prey on leafminer eggs while *Nabis punctipennis* Blanchard (Hemiptera: Nabidae) prey on both eggs and larvae. Freidberg and Gijswiit (1983) have recorded empidid and muscid flies attacking the adults.

1.2.6.3 Pathogens

Entomopathogens cause disease in insects through the effects of infection, parasitism and/or toxaemia (Lacey and Brooks, 1997). Some of the entomopathogens that have been reported to infect leafminers include nematodes, bacteria and fungi.

1.2.6.3.1 Entomopathogenic nematodes

A few species of entomopathogenic nematodes namely *Heterorhabditis* sp. (Heterorhabditidae: Rhabditida) and *Steinernema* sp. (Steinernematidae: Rhabditida) have been found infecting *Liriomyza* sp. (Williams, 1993; Walters *et al*., 2000). Nematodes are known to attack larvae, prepuparia and early puparia of leafminers (Liu *et al*., 2008).

1.2.6.3.2. Bacteria

There have been few trials with *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) for the control of agromyzid species (Çikman and Çömlekçioğlu, 2006; Çikman *et al*., 2008). Results on these studies revealed that application of *B. thuringiensis* at a concentration of 60 x 10^6/mg at the recommended rate of 75g/100 liters of water, reduced leafminer numbers in bean and chickpea fields in Turkey.

1.2.6.3.3 Fungi

The potential of entomopathogenic fungi as effective biological control agents for dipteran leafminers has been demonstrated. For instance, Bordat *et al*. (1988) tested the susceptibility of *L. trifolii* and *L. sativae* pupae to eleven strains of entomopathogenic Hypocreales (=Hyphomycetes) in the laboratory. *Liriomyza trifolii* was found to be susceptible to strains of *Isaria farinosus* (=*Paecilomyces farinosus*) (Holmsk.) strain 46 (Eurotiales: Trichocomaceae) (resulting in 23% emergence) and *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) (Wize) Brown and Smith strain 45 and 59 (resulting in 2.5 and 4% adult emergence respectively). *Liriomyza sativae* pupae were found to be less susceptible to the tested strains. *Metarhizium anisopliae* 78 and *I. farinosus* 46 were found to be

highly efficient restricting adult emergence to only 23.5% and 27.5% of pupae, respectively. In yet another study, Borisov and Ushchekov, (1997) found that *P. lilacinus* and *M. anisopliae* were effective in reducing adult emergence from soil by 70-94% and 60-88% respectively as compared with the untreated control.

1.2.7 Control of leafminers

1.2.7.1 Cultural control

Various cultural practices have been recommended for the control of leafminers. For instance, interplanting with field beans *Vicia faba* Linnaeus (Fabales: Fabaceae) was found to have potential for reducing damage by *L. trifolii* to chrysanthemums in glasshouses (Waterhouse and Norris, 1987). Cleaning up and burning of infested plant residues after harvesting and intercropping can substantially reduce populations in the following generations (Spencer, 1973; Weintraub and Horowitz, 1995).

1.2.7.2 Host plant resistance

Some plant varieties appear to be resistant to leafminer attack. For example, Musgrave *et al*. (1975) reported that celery variety #214 was highly attractive to adult *Liriomyza,* and the plant's leaves frequently were riddled with mines. Conversely, celery variety #16-24 was less attractive to adults and mines were far less frequent, although there was no evidence of antibiosis. Some varieties of potato known to be resistant to leafminer attack have been screened under field conditions in Peru (CIP, 1993).

1.2.7.3 Physical control

Yellow and green colours are known to attract leafminer adults, with yellow being the most common colour when sticky cards are used for monitoring (Chandler, 1981; Martin *et al*., 2005). The use of electrically powered backpack suction traps, mobile and stationary yellow sticky traps have been proved to effectively reduce leafminer adult populations (Price *et al*., 1981; Bennet, 1984). Webb and Smith (1970) proposed that maintaining chrysanthemum cuttings under normal

glasshouse conditions for 3-4 days after lifting to allow eggs to hatch and subsequent storage of plants at 0°C for 1-2 weeks should kill the larvae. Additionally, Gamma irradiation of eggs and first larval stages at doses of 40-50 Gy provided effective control (Yathom *et al*., 1991).

1.2.7.4 Chemical control

Pesticides such as broad-spectrum pyrethroids and organophosphates, nereistoxins and, to a lesser extent translaminar compounds such as Cyromazine and Abemectin are applied for control of leafminers (Rauf *et al*., 2000). In South Africa, Cyromazine is applied twice weekly on tomato seedlings to control *L. trifolii* on farmlands, and once weekly after planting or whenever a threshold of 0.25 mines per plant is reached (Kotze and Dennill, 1996). Botanical insecticides derived from the seed of the neem tree, *Azadirachta indica* Juss (Sapindales: Meliaceae) (Neemix-45) have also been used for control (Weintraub and Horowitz, 1997).

1.2.7.5 Biological control

Biological control is a preferred option for control of leafminers (Minkenberg and Van Lenteren 1986; Waterhouse and Norris, 1987; Johnson, 1993). In Hawaii for example, *Opius dissitus* Muesebeck (Hymenoptera: Braconidae), *Halticoptera patellana* Dalman (Hymenoptera: Pteromalidae), *Ganaspidium hunteri* Crawford (Hymenoptera: Eucoilidae) and the eulophids *Diglyphus begini* Ashmead, *Hemiptarsenus varicornis* Girault, *Derostenus fullawayi* Crawford, *Chrysocharis parksi* Crawford and *Closterocerus utahensis* Girault have been reported as parasitoids attacking vegetable leafminer larvae while they were feeding within the leaf tissue (Hardy and Delfinado, 1980). In Indonesia, the most important local parasitoids for leafminers include *Hemiptarsenus varicornis* Girault (Hymenoptera: Eulophidae), *Opius* spp. (Hymenoptera: Braconidae) and in some areas *Gronotoma micromorpha* Perkins (Hymenoptera: Eucoilidae) (Rauf *et al*., 2000).

1.2.8 Entomopathogenic fungi

There are more than 700 species of fungi from about 90 genera that are reported to be pathogenic to insects (Roberts and Humber, 1981; Charnley, 1989). Genera that have been mostly intensively investigated for development of mycoinsecticides include *Beauveria, Metarhizium, Lecanicillium* and *Isaria* since they are relatively easy to mass produce (Vega *et al*., 2009).

1.2.9 Infection process

Entomopathogenic fungi infect the host through the cuticle although infection through the digestive tract is also possible (e.g. *Ascophaera* and *Culicinomyces*) (Ferron, 1981; Goettel and Inglis, 1997; Goettel *et al*., 2000).

1.2.9.1 Spore attachment to the host cuticle

Pathogenicity begins with adhesion of the conidia to the host (Brobyn and Wilding, 1977; Zacharuk, 1970 a, b, c). Conidia initially attach via passive hydrophobic forces and sometimes by preformed mucilage. Permanent attachment is achieved by the joint action of mucilage and enzymes secreted actively prior to germ tube emergence (Boucias *et al*., 1988; Wraight *et al*., 1990). For *B. bassiana* and *M. anisopliae*, adhesion appears to be due to hydrophobic forces exerted by the rodlets covering the conidia (Charnley, 1989). The mucoid coating of *M. anisopliae* conidia is sparse, suggesting that its role in adhesion is weak and that there is a danger of loss until the appresorium has provided a solid anchorage (Zacharuk, 1970a).

1.2.9.2 Spore germination

A wide range of factors influence spore germination and behaviour. These include water, ions, fatty acids, nutrients, the biota on the cuticle surface, and the physiological state of the host insect cuticle (Butt *et al*., 1990; Dillon and Charnley, 1990). For mycopathogens to successfully infect their host insect, water is essential. Generally, relative humidity greater than 90% is needed for

germination (Robert and Campbell, 1977). Most Deuteromycetes have non-specific requirements for germination, but germination inhibitors may be present in the host (Brownlee *et al*., 1990).

1.2.9.3 Penetration of the cuticle

Penetration of the insect cuticle is through a combination of mechanical pressure and enzymatic degradation (Zacharuk, 1970c; Brobyn and Wilding, 1983). The enzymes responsible are lipases, proteinases and chitinases (Weiser, 1982). The germ tube may either penetrate directly into the cuticle or an appressorium may be formed which attaches firmly to the cuticle and a narrow infection peg sent into the cuticle (Zacharuk, 1970 a, b, c). The latter fungal structures are a prerequisite for infection for most entomopathogens and they form at the end of short germ tubes, sub-terminally or on side branches after extensive growth. It is known that colonies of entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* produce protease, lipase and chitinase in liquid and in agar (Gabriel, 1968), which help in cuticular invasion.

1.2.9.4 Growth of the fungus in the haemocoel and immune response of the host

The fungus usually grows in the haemocoel as yeast-like hyphal bodies often referred to as blastospores that multiply by budding during the pathogenic phase (Charnley, 1992) or wall-less amoeboid protoplasts (Goettel *et al*., 2000). Host defenses include a phenoloxidase system which deposits oxidized phenols (melanin) and protease inhibitors in the cuticle, and which may restrict pathogen enzyme activity (Moore and Prior, 1993). Within the haemocoel the main cellular defense against the fungus appears to be nodule formation, with haemocytes trapping fragments of fungus (Charnley, 1992). However, the entomopathogenic fungi overcome the defense system of the host insects by producing mycotoxins (Evans, 1989). These include destruxins and desmethyl-destruxin, which incite progressive degeneration of host tissues due to loss of structural integrity of membranes, followed by dehydration of cells as a result of fluid loss (Ferron,

1981). Several toxic compounds have been isolated and identified from cultures of *Beauveria* and *Metarhizium* (Roberts, 1969). However, many pathogenic fungi do not produce toxins (Moore and Prior, 1993). Some Entomophthorales form protoplasts, which do not contain the immune modulator B1, 3 glucans (which signals the presence of fungus to the host), in the haemocoel, and blastospores of Deuteromycetes reduce the effectiveness of cellular defences both by their rapid production in vast numbers and by being less antigenic than mycelia (Moore and Prior, 1993).

1.2.9.5 Growth in the mycelial phase with invasion of virtually all organs of the host

Host death marks the end of the parasitic phase of fungal development after which the fungus grows saprophytically through all the tissues. It penetrates the integument and develops conidiophores on the cuticle surface only when the atmosphere is saturated with water (Ferron, 1981). With *Metarhizium* and *Beauveria*, the insects often die with their legs extended and fall from plants. Following death, fungal hyphae may appear through the integuments of the cuticle and sporulation of the fungus may occur (Hill, 1994).

1.2.10 Factors affecting efficacy of fungi as biological control agents

A complex of interacting processes, both environmental and biotic, is necessary for or inhibitory to the development of epizootics caused by entomopathogenic fungi. These include sensitivity to solar radiation, microbial antagonists, host behavior, physiological condition and age, pathogen vigor and age, presence of pesticides and appropriate temperature, humidity and inoculum thresholds (McCoy *et al*., 1988; Ferron *et al*., 1991; Hajek and St. Leger, 1994; Lacey and Goettel, 1995).

1.2.10.1 Environmental factors

1.2.10.1.1 Temperature

Temperature is an important abiotic factor affecting the efficacy of entomopathogenic fungi (Carruthers *et al*., 1985; Benz, 1987; Ferron *et al*., 1991; Watanabe, 1987). Most entomopathogenic fungi have a wide range of temperature tolerances (i.e. 0-40^{0}C), however, temperature optima for infection, growth and sporulation are usually much more restricted (generally 20-30^{0}C) (Goettel *et al*., 2000). Quedraogo *et al.* (1997) reported that optimal temperatures for 22 isolates of *M. anisopliae* and 14 isolates of *M. flavoviride* was generally between 25 and 32^{0}C with several isolates exhibiting optimal growth at temperatures as high as 32^{0}C. In this study, the optimal temperature for the majority of *M. anisopliae* isolates was found to be 25^{0}C. Some authors have also reported an optimal growth temperature of 25^{0}C for some *M. anisopliae* isolates (Fargues *et al.*, 1992; Dimbi *et al*., 2004). Thermal constraints are not only the result of ambient conditions, but also influenced by host thermoregulation (Quedraogo *et al*., 1997). Some insects can elevate their body temperature either through habitat selection or basking in the sun and such activity has been shown to reduce disease incidence of *Entomophthora muscae* Cohn (Entomophthorales: Entomophthoraceae) in houseflies (Watson *et al*., 1993). It has also been shown that acridids can raise their temperatures to above 40^{0}C in response to infection by *B. bassiana* (Inglis *et al*., 1996) or *M. flavoviride* (Inglis *et al*., 1997) thereby inhibiting and/or preventing disease caused by the two pathogens.

1.2.10.1.2 Relative humidity

Relative humidity is often the limiting factor for the activity of pathogenic fungi (Fargues and Remaudiere, 1977). Fungal sporulation and spore germination require free water or humidity of at least 90% (Goettel *et al*., 2000; Moore and Prior, 1993). Daoust and Roberts (1983) reported that conidia of *M. anisopliae* survived best when RH was high (97%) at moderate temperatures (19-27^{0}C). However, not all fungi require high moisture for infectivity. For instance, *M.*

flavoviride is capable of infecting the desert locust, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) at relative humidities as low as 13% (Fargues *et al*., 1997). Moisture can also have very significant effects on the persistence of fungal inocula (Goettel *et al.,* 2000).

1.2.10.1.3 Solar radiation

The ultraviolet radiation (uv-B; 280-320 nm) component of sunlight is detrimental to all microorganisms (Tevini, 1993). It causes primary (i.e. nucleic acid mutations) and/or secondary (i.e. photoreactions) damage to exposed microorganisms, either of which may lead to cellular death. However, there are differences in susceptibility to irradiation between entomopathogenic fungal species and among strains within species (Fargues *et al*., 1996; Goettel *et al*., 2000).

1.2.10.2 Biotic factors

1.2.10.2.1 Host plant

The inter and intra-specific variation in host plants has been shown to affect herbivore survival, growth, reproduction, dispersal (Price *et al*., 1980; Denno and McClure, 1983; Cory and Hoover, 2006) and susceptibility to disease (Tanada and Kaya, 1993; Poprawski *et al*., 2000; Ugine *et al*., 2007). The host plant of phytophagous insects can significantly affect their susceptibility to disease either through dietary stress or direct antimicrobial activity of the plant (Tanada and Kaya, 1993; Cory and Hoover, 2006). Many plants produce antimicrobial compounds, which inhibit the activity of entomopathogens (Costa and Gaugler, 1989; Lacey and Mercadier, 1998; Poprawski *et al*., 2000). Insect herbivores are also known to sequester antimycotic phytochemicals that help confer resistance to fungal infection (Poprawski *et al*., 2000).

1.2.10.2.2 Host insect factors

Among host factors, host behaviour (Fuxa, 1987), host density (Watanabe, 1987), host age, host species, developmental stage and sex have been reported to affect insect susceptibility to entomopathogenic fungi (Ferron, 1985; Maniania

and Odulaja, 1998; Dimbi *et al.*, 2003). Behaviour such as grooming, cannibalism, aggregation patterns, level of activity, removal of infected by uninfected hosts, and feeding in protected situations are known to affect whether hosts become infected (Benz, 1987; Kaya, 1987; Maddox, 1987; Watanabe, 1987). Changes in behaviour after infection such as changes in flight habits or capability, feeding during daylight rather than at night, movement to unusually high positions on host plants and seclusion in debris are thought to be important in epizootiology (Fuxa, 1987).

1.2.10.2.2.1 Host developmental stage and sex

The development stage and sex of the insect affects the efficacy of entomopathogenic fungi and not all stages in the insect lifecycle are equally susceptible (Tanada, 1963; Maniania and Odulaja, 1998; Dimbi *et al*., 2003). In a study of the effect of species, age and sex of tsetse (*Glossina morsitans morsitans* Westwood and *G. m. centralis* Machado (Diptera: Glossinidae)) on response to infection by *M. anisopliae*, host age was found to have a pronounced effect on susceptibility while the females of both species were more susceptible than males (Maniania and Odulaja, 1998).

1.2.10.2.3 Pathogen properties

Pathogen properties such as host specificity (Fuxa, 1987), pathogen virulence, infectivity, persistence, and the capacity to disperse are key factors affecting the ability of entomopathogens to produce epizootics (Tanada, 1963; Jenkins and Goettel, 1997).

Host specificity governs the ability of pathogenic microorganisms to infect potential insect hosts and develop and reproduce within them (Hajek *et al*., 1995). Some fungi have restricted host ranges like *Aschersonia aleyrodis* Webber (Clavicipitaceae: Hypocreales) infects only whiteflies and soft scales, while others have a wide host range at the species level although individual isolates may be selective (Samson *et al.*, 1988). *Metarhizium anisopliae* has

been identified from about 300 species of Lepidoptera, Coleoptera, Orthoptera and Hemiptera while *B. bassiana* has over 700 recorded host species (Moore and Prior, 1993). However, isolates of *Metarhizium* spp. and *Beauveria* spp. show host ranges which are usually restricted to within the order of the original host, and sometimes even more narrowly, to its family (Moore and Prior, 1993). *Metarhizium anisopliae* has been shown to produce penetration structures (appressoria) in response to cuticular surface topography of hosts (St. Leger *et al*., 1991). In particular, appressoria were frequently formed around bases of setae of *Manduca sexta* Linnaeus (Lepidoptera: Sphingidae). Conidia of *Nomuraea rileyi* (Farlow) Samson (Clavicipitaceae: Hypocreales) have been shown to orientate to and penetrate the membranous regions surrounding cuticular spines on *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) larvae (Boucias and Pendland, 1991). Greater virulence results in better short-term control of insect populations (Tanada and Fuxa, 1987).

1.2.11 Strategies for microbial control of insect pests

The use of entomopathogenic fungi for biological control follows different strategies: permanent introduction and establishment, inundative/inoculative augmentation, environmental manipulation/conservation and auto-dissemination (Fuxa, 1987).

1.2.11.1 Introduction

This strategy involves the establishment of an organism in a pest population where it does not naturally occur and which results in more or less permanent suppression of the pest (Hamm, 1984). Burges and Hussey (1971) and Burges (1981) cited a total of 41 successful introductions of insect pathogens. The most notable are those of *Paenibacillus popilliae* (=*Bacillus popilliae*) Dutky (Bacillales: Paenibacillaceae) for control of *Popillia japonica* Newman (Coleoptera: Scarabaeidae) (Klein, 1981) and nucleopolyhedrovirus (=nuclear polyhedrosis virus) (NPV) for control of *Gilpinia hercyniae* Hartig (Hymenoptera: Diprionidae) (Cunningham and Entwistle, 1981). The pathogen *Entomophaga maimaiga*

Humber (Entomophthorales: Entomophthoraceae) is known to cause epizootics in *Lymantria dispar* Linnaeus (Lepidoptera: Lymantriidae) populations (Hajek *et al.*, 1995). This pathogen was first introduced into the Northeastern United States during 1910 and 1911 for control of *L. dispar* (Lacey *et al*., 2001).

1.2.11.2 Augmentation

There are two approaches to augmentation: inundative and inoculative. Inoculation involves release of relatively small amounts of pathogen with the expectation that the pathogen will establish in the target population and spread (Goettel and Jaronski, 1997). Inundative release involves the mass application of a pathogen on a regular or periodic basis (Goettel and Jaronski, 1997).

1.2.11.3 Conservation

Conservation or manipulation of the environment (ecosystem) involves the enhancement of naturally occurring pest control by means other than direct addition to the pathogen units already present (Nordlund, 1984). Cultural manipulation can permit the pathogen to reproduce more than usual or can preserve or enhance those already present (Fuxa, 1987). Elimination of fungicide treatments for example has been used to enhance *N. rileyi* epizootics (Johnson *et al*., 1976).

1.2.11.4 Auto-dissemination

This is a new approach, which involves strategies that manipulate a proportion of the target insect population to facilitate the dispersal of a pathogen to its wider pest population (Hunter-Fujita *et al*., 1998; Vega *et al*., 2000). Combining the standard Japanese beetle trap containing floral lure, and an autoinoculating chamber containing fungal spores, Klein and Edwards (1989) were able to attract and infect large numbers of male and female beetles. Furlong and Pell (2001) observed an efficient horizontal transmission of conidia of *Zoophthora radicans* Brefeld (Batko) (Entomophthorales: Entomophthoraceae) to the diamondback moth. Male moths were attracted into a sex pheromone trap and inoculated with

the fungus, which is known to frequently cause natural epizootics in diamondback moth populations (Ooi, 1981; Yamamoto and Aoki, 1983).

1.2.12 Autoinoculator

To promote assisted autodissemination, a device known as an autoinoculator is used. Autoinoculators can target control agents to particular pests or diseases, and can be constructed cheaply from locally available material (Vega *et al*., 2000). Maniania (2002) and Dimbi *et al*. (2003) showed that tsetse and fruit flies (*Ceratitis* spp.) (Diptera: Tephritidae) could be used to vehicle fungal conidia of *M. anisopliae* from a contamination device. Flies treated that way lived for a few days, and were able to contaminate healthy flies with fungal spores during mating and thus introduce the disease into the field population. Examples of manipulated dissemination of entomopathogens by insects using autoinoculator devices are listed by Vega *et al.* (2000).

1.2.13 Safety of entomopathogenic fungi

Biological control, whether classical or conservation, relies on the recognition, understanding and appreciation of the action of natural enemies (Ooi, 2000). One of the major objectives of biological control is the demonstration of specific techniques for limiting the rapid multiplication of pest without significantly perturbing the other organisms in the biocoenosis (Hurpin, 1973 cited in Fargues and Remaudiere, 1977). Mycoinsecticides have features that provide ecologically sounder pest control than chemical pesticides (Moore and Prior, 1993). They are selective to varying degrees, often suitable for integrated pest management techniques, may provide an extended period of control by remaining within the environment (or even establish permanently) and are biodegradable (Goettel and Johnson, 1992). Although fungal biocontrol agents are considered environmentally benign, the greatest concern is their effect to non-target organisms in the form of toxicity, allergy and direct infection (Austwick, 1980). Goettel *et al*. (1990) reported that the use of a mycoinsecticide could lead to host depletion which could result in a reduction of the populations of other natural

enemies such as parasitoids and predators. Van den Berg (1990) reviewed the safety of four entomopathogens on caged honey bees and determined that the pathogen *E. maimaiga* and the bacterium *B. thuringiensis* were safe to caged adult honey bees while *B. bassiana* was found to reduce longevity and cause mycosis among treated bees.

1.3 Justification of study

Entomopathogenic fungi which infect their host through the cuticle offer a better alternative for control of sap-feeding insects (Poprawski *et al*., 2000; Inbar and Gerling, 2008). Various strains of the hyphomycetous fungi, *M. anisopliae* (Metchnikoff) Sorokin and *B. bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) have been reported to be virulent to other dipteran pests (Watson *et al*., 1995; Maniania and Odulaja, 1998; Quesada-Moraga *et al*., 2006). However, their use in dipteran leafminer management has been limited to the screening of fungal isolates against puparia in the laboratory and not to adults (Borisov and Ushchekov, 1997; Bordat *et al*., 1988).

The general aim of this study was therefore to explore the use of entomopathogenic fungi as a component of leafminer management.

The specific objectives of this study were addressed under the following topics which are each addressed in a separate chapter of the thesis:

a) Pathogenicity of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) isolates to the adult pea leafminer *Liriomyza huidobrensis* (Diptera: Agromyzidae) and prospects of an autoinoculation device for infection in the field

b) 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

c) Effect of constant temperatures and host plant on the virulence of *Metarhizium anisopliae* isolates to three species of adult leafminers, *Liriomyza huidobrensis, Liriomyza trifolii* and *Liriomyza sativae*

d) Effect of the entomopathogenic fungus *Metarhizium anisopliae* on the leafminer ectoparasitoid *Diglyphus isaea*.

The above mentioned objectives are each discussed below. Each chapter was prepared in manuscript format.

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CHAPTER TWO

2. 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

2.1 Abstract

Seventeen isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin and three isolates of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) were evaluated for their pathogenicity to the adult pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), in the laboratory. Flies were contaminated with dry conidia through a velvet material wrapped around the inner side of a cylindrical plastic tube. All the isolates were pathogenic to the pea leafminer causing mortality between 40 and 100% at five days post-exposure. The lethal time for 50% mortality (LT_{50}) ranged from 2.6 to 5.4 days while the LT_{90} values varied between 3.2 and 9.1 days depending on the isolate. An autoinoculation device was evaluated in cage field experiments using only one of the virulent isolates, *M. anisopliae* ICIPE 20. The device was loaded with 2-3g of dry conidia. Mortality of up to 100% was observed in flies captured from fungus-treated cages held under laboratory conditions. The average number of spores picked up by a single fly visiting the device increased with days after inoculation. One day after the inoculation, adults picked-up an average of 4.1 ± 0.7 x 10^5 conidia and 39.6 ± 4.0 x 10^5 conidia 5 days post-inoculation. Depending on the sampling date, the LT_{50} varied between 1.8 and 3.4 days. Results indicate that some isolates of *B. bassiana* and *M. anisopliae* are highly pathogenic to *L. huidobrensis* suggesting a potential for their use in the control of this pest. They also suggest the possibility of *L. huidobrensis* suppression with fungi using an autoinoculation device.

Published as: MIGIRO, L., MANIANIA, J., OLAYE, C.A & VAN DEN BERG, J. 2010. Pathogenicity of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Hypocreales: Clavicipitaceae) to the adult pea leafminer *Liriomyza huidobrensis* (Diptera: Agromyzidae) and prospects of an autoinoculation device for infection in the field. Environmental Entomology 39, 468–475.

2.2 Introduction

The pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), is a highly polyphagous and invasive pest responsible for major yield losses in flower and vegetable crops (Spencer, 1973; Weintraub and Horowitz, 1995; Wei *et al*., 2000; Martin *et al*., 2005a) recorded attacking at least 14 plant families (Spencer, 1990). Adults damage crops by puncturing the leaf surface to feed and lay eggs into the tissue. When the eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines (Spencer, 1973; Knodel-Montz *et al*., 1985; Ameixa *et* al., 2007). Leaf mines and punctures reduce the photosynthetic ability and the quality of high value horticultural crops (Spencer, 1973, 1990; Kox *et al*., 2005). During outbreaks, severe damage resulting from both adult puncturing and larval-mining can lead to total crop losses (Spencer, 1973, 1990). Leafminers are also listed as quarantine pests in the EU Plant Health Directive 2000/29 (EU 2000), hence in addition to the direct damage, losses also result from the restriction in trade and export markets.

The management of leafminers by both small and large-scale producers worldwide has commonly relied on the use of chemical insecticides (Murphy and LaSalle, 1999). However, the indiscriminate and frequent use of these chemicals has resulted in insecticide resistance of flies as well as elimination of their natural enemies (MacDonald, 1991; Weintraub and Horowitz, 1995; Murphy and LaSalle, 1999; Rauf *et al*., 2000). Other leafminer non-chemical control methods which include using parasitoids (Minkenberg and van Lenteren, 1986; Waterhouse and Norris, 1987; Johnson, 1993), trapping by yellow sticky traps (Price *et al*., 1981; Bennett, 1984), resistant plant varieties (CIP, 1993), entomopathogenic nematodes (Walters *et al*., 2000) and bacteria (Çikman and Çömlekçioğlu, 2006; Çikman *et al*., 2008) have been attempted with varied levels of success.

Entomopathogenic fungi which infect their host through the cuticle offer a better alternative for sap-feeding insects (Poprawski *et al*., 2000; Inbar and Gerling,

2008). Various strains of the hyphomycetous fungi, *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) have been reported to be virulent to other dipteran pests including house fly (Watson *et al*., 1995; Renn *et al*., 1999), tsetse fly (Kaaya and Okech, 1990; Maniania,1998) and fruit flies (Dimbi *et al*., 2003; Quesada-Moraga *et al*., 2006). There have been few attempts to use entomopathogenic fungi against dipteran leafminers (Borisov and Ushchekov, 1997; Bordat *et al*., 1988) but these studies were limited to the screening of fungal isolates in the laboratory against puparia and not adults. The major challenge for using mitosporic entomopathogenic fungi to control dipteran flies has been their application in the field. Recently, contamination devices were developed to infect fruit flies (Dimbi *et al*., 2003) and tsetse flies (Maniania, 1998; Maniania, 2002) and tested in the field with success (Maniania *et al*., 2006; Ekesi *et al*., 2007). Therefore, this technique offers new prospects for application of entomopathogenic fungi for the control of dipteran leafminers.

Contamination devices use visual and chemical cues to attract tsetse flies and fruit flies respectively. The leafminer fly trapping technique using yellow sticky cards has been based on visual cues only, because no known sex pheromone has been identified or isolated. Previous leafminer studies when sticky cards were used have shown an attraction for yellow and green, with yellow being the most preferred color (Chandler, 1981; Zoebisch and Schuster, 1990; Martin *et al*., 2005b). In fact, yellow color trap boards coated with tangle foot and electrically driven backpack suction traps have been used to monitor and reduce leafminer populations (Bennett, 1984; Price *et al*., 1981). The design of a device that will attract leafminers to the foci of inoculum will be of paramount importance in ensuring field fly contamination thereby enhancing control using fungus.

The objectives of this study were 1) to screen fungal isolates of *Beauveria* and *Metarhizium* for their virulence against the pea leafminer in the laboratory in order to select the most virulent strain that could be developed as biocontrol

agents, and 2) evaluate the performance of an autoinoculation device for infecting adult flies in the field.

2.3 Materials and methods

2.3.1 Insects

Adults of *L. huidobrensis* were obtained from the mass rearing unit at the International Centre of Insect Physiology and Ecology (*icipe*). The leafminer colonies were maintained on faba bean, *Vicia faba* L., in a rearing room (25-27°C, 60-80% RH with a 12 L: 12 D photoperiod). Adults were fed on 10% sugar solution from balls of cotton wool soaked in the solution placed at the bottom corner of the rearing cages. In all the bioassays, 1 to 2 day-old adult flies were used.

2.3.2 Fungal isolates

The 20 fungal isolates (17 of *M. anisopliae* and 3 of *B. bassiana*) used in the study were obtained from *icipe* Arthropod Germplasm Centre (Table 1). The isolates were cultured on Sabouraud dextrose agar (SDA) in Petri dishes (9 cm) and incubated at 25 ± 2°C in complete darkness. Conidia were harvested from 3 week-old cultures with a sterile spatula. The viability of conidia was based on germ tube formation (Goettel and Inglis 1997). Conidial suspensions (0.1 ml) titrated to 3 x 10^6 conidia ml^{-1} were spread-plated on Petri dishes (9 cm) containing SDA medium. A sterile microscope cover slip (2 x 2 cm) was placed on top of the agar in each plate. Plates were incubated in complete darkness at 25 ± 2°C and examined after 20 hours. Percentage germination of conidia was determined by assessing whether a germ tube (twice the diameter of the propagule) had formed in 100 random conidia on the surface area covered by each coverslip under the light microscope (400X) (Goettel and Inglis, 1997). Four replicate plates per isolate were used.

2.3.2.1 Pathogenicity of *M. anisopliae* and *B. bassiana* isolates against adult *L. huidobrensis.*

A modified contamination technique described by Dimbi *et al*. (2003) for fruit flies was used. Adults of *L. huidobrensis* were exposed to 0.1g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (70 mm length × 48 mm diam). For each isolate, 25 flies were transferred into the cylindrical tube and allowed to walk on the velvet for 1 min, after which 20 insects were transferred from the velvet into a clean ventilated Perspex cage (150 x 150 x 200 mm). A ball of cotton wool soaked in 10% sugar solution was placed at the bottom corner of the cage as a food source. Insects in the control treatments were exposed to fungus-free velvet cloth before being transferred into similar cages. Treatments were arranged in a complete randomized design and repeated four times. Flies were maintained at 25-27°C, 50-70% RH under a 12L: 12D photoperiod.

Mortality was recorded daily for 11 days. Dead insects were surface sterilized in 70% alcohol followed by three rinses in distilled water and transferred to Petri dishes lined with damp sterilized filter paper to allow fungal growth on the surface of the cadaver. Dead flies that showed fungal growth/sporulation on the surface of the cadavers were used for analysis. Five flies that had been left out of the initial 25 were used to estimate the number of conidia picked up by a single fly in each treatment. Insects were transferred 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 was determined using a hemocytometer.

2.3.3 Mass production of selected fungal isolate

One of the most virulent isolates, *M. anisopliae* ICIPE 20, was selected for field cage experiments to evaluate the performance of an autoinoculation device. Conidia were mass-produced on whole rice substrate in Milner bags (60 cm long x 35 cm wide). Rice was autoclaved for 1 h at 121°C and inoculated with a three

day-old culture of blastospores (Jenkins *et al*., 1998). The sterile rice was then incubated for 21 days at 20-26°C, 40-70% RH and then allowed to dry for five days at room temperature. Conidia were then harvested by sifting the substrate through a sieve (295-µm mesh size). The conidia were stored at 4-6°C until used. Viability was determined as described above and germination of 86-90% was recorded after 20 h.

2.3.3.1 Description of autoinoculation device

After preliminary studies, two types of autoinoculation devices were selected and evaluated for their ability to attract and infect flies. The first prototype was made from 500-ml clear disposable plastic beverage bottles (5.5 cm diam, 21 cm in height), which will henceforth be called device A. Five entry/exit holes (2 × 3 cm) were made near the top and another set of five near the bottom of the bottle at alternate positions using a pen knife. A dark grey cotton velvet cloth (3.5 diam × 11 cm height) was used to hold the conidia. It was held in place by gluing it on its smooth side and wrapping it around a smaller plastic diameter bottle (3 cm diam and 11.5 cm height) exposing the rough surface. To attract the adult leafminers, a yellow netting (3.8 cm diam × 11 cm height) of 1 mm^2 holes was wrapped around the velvet cloth and tightened with two office pins (Flyingdeer Office Pins No. 2, Zheijiang Flyingdeer Industrial Co., Ltd, Zheijiang, China) to ease cleaning and replenishing of conidia. The smaller plastic diameter bottle was later introduced into the bigger bottle and fixed at the center using a metallic wire which also served for hanging the device. The second device was a modification of the Lynfield trap (11 cm diam × 10 cm height) (Figure 2.1). Holes similar to the ones described above were made on the trap (Figure 2.1, B1) and velvet (8 cm diam × 8.5 cm length) and yellow netting (8.9 cm diam × 8.6 cm length) wrapped around a smaller inner cylindrical bottle (5.2 cm diam and 6 cm in height) (Figure 2.1, B2) that was then hung in the trap as described above. This device will henceforth be called device B (Figure 2.1, B3).

2.3.3.2 Selection of autoinoculation device

All faba bean plants used in this study were planted in screen houses (2.8 m length x 1.8 width × 2.2 m height) in 15 cm pots (5-8 plants per pot), using a mixture of manure and soil in a ratio of 1:5. After two weeks, 20 potted plants (ca. 35 cm in height) were transferred to field cages (2 m height × 2.9 m diam) where they were arranged in four rows (30 cm inter and 15 cm intra-row spacing). Tangle foot glue (Tangle-trap, The Tanglefoot Company, Michigan, USA) was smeared around the yellow netting of the autoinoculation devices. These were then suspended in the field cage 1m apart each at canopy level between the two center rows of the bean plants using a string. One hundred 1-2 d-old flies were then released into the cage. After 8 h, the number of trapped flies was recorded. Treatments were carried out simultaneously in two field cages and the experiment was replicated four times.

2.3.3.3 Evaluation of autoinoculation device in field cage

The performance of device B was assessed in a field cage (2 m height × 2.9 m diam) between 24 January and 19 February 2009 to determine its efficiency in delivering inoculum into the leafminer population. Four rows of 20 potted two week-old faba bean plants (height of 35 cm) grown as described under 2.3.3.2 were placed in a field cage a day prior to the experiment. Approximately 2-3g of conidia was spread evenly on the velvet cloth of the autoinoculation device. The yellow netting was then wrapped around the velvet cloth containing conidia and tightened with two office pins. The inner side of the device and the outer side of the yellow netting were also lightly dusted with conidia (using a camel brush) to maximize contamination. The device was then hung as described earlier and suspended at canopy level (35 cm) at the center between the two middle rows of the bean plants using a string. Five hundred 1-2 d-old flies were then released into the field cage. A similar trap device without fungus was suspended in another field cage and acted as the control. At 1, 2, 3, 4 and 5 d after exposure, 30 live flies were collected (from each of the two field cages) at random and transferred individually into clean sterile cylindrical plastic tubes (diam 7 mm ×

height 49 mm) and brought to the laboratory. Twenty of the 30 tubes containing flies were maintained in an incubator at 25 ± 2°C and 80-90% RH. The mouth of the tube was replaced with a plug of cotton wool soaked in 10% sugar solution as a food source, which was replaced with fresh ones every second day. Ten of the remaining tubes with flies were used to estimate the number of conidia picked up by a single fly in each day after exposure using the procedure described earlier. The experiment was replicated four times. Mortality was recorded daily until all the flies died. Dead flies were surface sterilized as described earlier and only mycosed flies were used in the analysis.

The number of pupae produced at the end of the experiment was determined from six potted plants randomly picked from the treatment cages. The potted plants were transferred into rearing cages (0.6 x 0.5 x 0.6 m) until mines were mature. Leaves containing mature mines were then harvested and placed on a wire mesh over a tray containing 100g of sterile sand spread evenly at the bottom of the container to collect pupating larvae or pupae falling off the leaves. The number of pupae was recorded. The same procedure was used for the control treatment.

a

b

c

Plate 2. 1 *Beauveria bassiana* (a), *Metarhizium anisopliae* (b) isolates growing on Sabouraud Dextrose Agar and contamination tube lined with velvet material used to infect insects with spores (c).

2.3.4 Statistical analysis

Percent mortality and germination were corrected for control mortality (Abbott 1925) and normalized by arcsine transformation (Sokal and Rohlf, 1981) before being subjected to analysis of variance (ANOVA) using PROC GLM, at 95% level of significance. Student-Newman-Keul's (SNK) analysis was used to separate the means as a post ANOVA procedure (α = 0.05). Non-transformed means are presented in the tables. The LT_{50} and LT_{90} values were determined for each replicate using the probit analysis method for correlation data (Throne *et al*., 1995) and compared among themselves using ANOVA (α = 0.05) and means separated using SNK. Correlation analysis was carried out to relate mortality rate with mean number of conidia picked up by a single fly from the contamination tube. Repeated measures analysis (PROC GLM-Wilks' Lambda statistic) was used to relate the number of spores picked up by a single fly from the autoinculation device to the different sampling days. Paired *t*-test was used to compare the performance of the two devices. The number of pupae produced in the treatments and those in the control were analysed using Wilcoxon-Mann-Whitney U-test. All analyses were performed using the SAS (SAS, 2003) version 9.1 statistic package.

2.4. Results

2.4.1 Pathogenicity of *M. anisopliae* and *B. bassiana* against adult *L. huidobrensis*

Germination of conidia of different fungal isolates used in the study ranged from 83-99% (Table 2.1).

Table 2.1 Identity of fungal isolates used in the study and their percent germination on SDA at 22-29^{0}C.

Fungal species	Isolate	Source	Locality/Country	Year of isolation	% Germination ± SE
Metarhizium anisopliae	ICIPE 18	Soil	Mbita (Kenya)	1989	97.0 ± 1.1abc
	ICIPE 20	Soil	Migori (Kenya)	1989	99.3 ± 0.5a
	ICIPE 40	Soil	Kitui (Kenya)	1990	86.8 ± 2.0e
	ICIPE 41	Soil	Lemba (D.R. Congo)	1990	97.8 ± 0.9abc
	ICIPE 51	Soil	Embu (Kenya)	1989	91.5 ± 1.6cde
	ICIPE 57	Soil	Kericho (Kenya)	2005	92.8 ± 1.1bcde
	ICIPE 60	Soil	Kakello (Kenya)	1990	98.3 ± 1.4ab
	ICIPE 62	Soil	Matete (D.R. Congo)	1990	97.0 ± 1.7abc
	ICIPE 63	Soil	Matete (D.R. Congo)	1990	94.5 ± 1.4abcde
	ICIPE 69	Soil	Matete (D.R. Congo)	1990	98.5 ± 0.7ab
	ICIPE 30	*Busseola fusca*	Kendubay (Kenya)	1989	96.5 ± 1.6abc
	ICIPE 78	*Temnoschoita nigroplagiata*	Ungoe (Kenya)	1990	85.8 ± 2.0e
	ICIPE 84	*Ornitacris turbida cavroisi*	Kaffraine (Senegal)	2003	96.0 ± 0.7abcd
	ICIPE 402	Homoptera	Shimba Hills(Kenya)	2007	82.5 ± 2.2e
	ICIPE 315	*Tetranychus urticae*	Kerugoya (Kenya)	2006	96.3 ± 2.2abc
	ICIPE 387	*Forficula senegalensis*	Mai Mahiu (Kenya)	2007	99.0 ± 0.6a
	ICIPE 07	*Rhipicephalus appendiculatus*	Rusinga Island (Kenya)	1996	97.5 ± 0.7abc
Beauveria bassiana	ICIPE 273	Soil	Mbita (Kenya)	2006	97.8 ± 1.3abc
	ICIPE 279	Coleopteran larvae	Kericho (Kenya)	2005	85.3 ± 0.9e
	ICIPE 603	Hymenoptera	Taita (Kenya)	2007	87.8 ± 3.2de

Within column, means followed by the same letters are not significantly different (Student-Newman-Keuls (SNK), α = 0.05). n = 80.

The number of conidia picked up by a single fly from the contamination tube was significantly different among the isolates ($F = 9.02$; $df = 19,80$; $P < 0.0001$) and ranged between 4.4×10^4 and 15.6×10^4 conidia per fly (Table 2.2). There was a positive correlation between mortality rates and the amount of conidia picked by a single fly for the different isolates ($r = 0.67$; $N = 84$; $P < 0.0001$). The mean mortality in the controls was 6.3 ± 1.3% five days after treatment. Both species of fungi were pathogenic to adult *L. huidobrensis*; but mortality varied significantly among the isolates ($F = 54.17$; $df = 20,63$; $P < 0.0001$) five days after infection (Table 2.2). Mortality ranged between 39.9% for the least virulent isolate (*M. anisopliae* ICIPE 51) and 100% for the most virulent isolates (*M. anisopliae* ICIPE 18 and *B. bassiana* ICIPE 273). Mortalities of adult *L. huidobrensis* inoculated with *B. bassiana* species did not differ significantly, and the average ranged between 93-100%. The lethal time to 50% mortality (LT_{50}) values were significantly different among the isolates ($F = 64.64$; $df = 15,48$; $P < 0.0001$) and ranged between 2.6 and 5.2 days. Similarly, the lethal time to 90% mortality (LT_{90}) values were significantly different among the isolates ($F = 33.49$; $df = 15,48$; $P < 0.0001$) and ranged between 3.2 and 9.1 days (Table 2.2).

Table 2.2 Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* isolates against *Liriomyza huidobrensis* adults. Percent mortality, lethal time mortality (LT_{50} and LT_{90}) (X ± SE) five days post-inoculation and the mean number of conidia per fly in the laboratory.

Fungal species	Fungal isolate	% Mortality (X ± SE)	LT_{50} values (X ± SE)	LT_{90} values (X ± SE)	Conidia picked up by a single fly x 10^4 ± SE
Metarhizium anisopliae	ICIPE 18	100a	2.9 ± 0.16hi	3.2 ± 0.1f	12.6 ± 1.4ab
	ICIPE 387	98.6 ± 1.4a	3.3 ± 0.02fg	4.2 ± 0.2ef	8.6 ± 0.6abc
	ICIPE 20	97.4 ± 1.5ab	2.8 ± 0.1hi	3.8 ± 0.2ef	12.5 ± 1.2ab
	ICIPE 84	97.4 ± 1.5ab	2.6 ± 0.04i	3.5 ± 0.1f	15.6 ± 3.0a
	ICIPE 62	96.1 ± 1.3ab	2.8 ± 0.1hi	4.1 ± 0.2ef	9.1 ± 0.6abc
	ICIPE 60	94.6 ± 2.3ab	3.0 ± 0.1gh	3.9 ± 0.6ef	9.0 ± 1.0abc
	ICIPE 07	85.4 ± 2.5ab	3.3 ± 0.1fg	5.3 ± 0.2cd	6.3 ± 0.5cd
	ICIPE 78	82.5 ± 3.7ab	3.4 ± 0.1ef	5.8 ± 0.5c	5.6 ± 0.9cd
	ICIPE 69	78.6 ± 2.5ab	4.0 ± 0.1c	5.9 ± 0.2c	4.7 ± 0.5d
	ICIPE 315	76.0 ± 1.8b	3.5 ± 0.1def	6.0 ± 0.4c	4.5 ± 0.7d

Table 2.2 (continued)

Fungal species	Fungal isolate	% Mortality (X ± SE)	LT_{50} values (X ± SE)	LT_{90} values (X ± SE)	Conidia picked up by a single fly x 10^4 ± SE
Metarhizium anisopliae	ICIPE 57	57.2 ± 4.9c	4.7 ± 0.1b	7.4 ± 0.3b	4.7 ± 0.6d
	ICIPE 63	55.8 ± 6.2c	4.5 ± 0.1b	7.6 ± 0.3b	4.6 ± 0.5d
	ICIPE 40	51.8 ± 6.9cd	5.2 ± 0.3a	9.1 ± 0.5a	4.4. ± 0.6d
	ICIPE 30	49.1 ± 5.8cd	-	-	4.5 ± 0.4d
	ICIPE 41	47.9 ± 3.2cd	-	-	4.7 ± 0.5d
	ICIPE 402	45.3 ± 2.1cd	-	-	4.4 ± 0.9d
	ICIPE 51	39.9 ± 3.1d	-	-	5.3 ± 0.3cd
	ICIPE 273	100a	2.9 ± 0.1hi	3.8 ± 0.2f	7.7 ± 0.6bcd
Beauveria bassiana	ICIPE 603	93.4 ± 1.3ab	3.7 ± 0.1cde	4.6 ± 0.1de	6.1 ± 1.2cd
	ICIPE 279	93.3 ± 2.6ab	3.8 ± 0.1cd	4.7 ± 0.3de	8.0 ± 1.1bcd
	Control	6.3 ± 1.3e	-	-	-
		n = 84	n = 64	n = 64	n = 100

Within columns, means followed by the same letters are not significantly different, ANOVA and Student-Newman-Keuls (SNK) comparisons of means, $\alpha = 0.05$.

2.4.2 Selection of autoinoculation device

Device B (Figure 2.1) made from the modified Lynfield trap attracted more flies (49.0 ± 11.7) than did the device A (15.9 ± 4.7) ($t = 8.23$; $P < 0.0001$) and was subsequently selected for further studies.

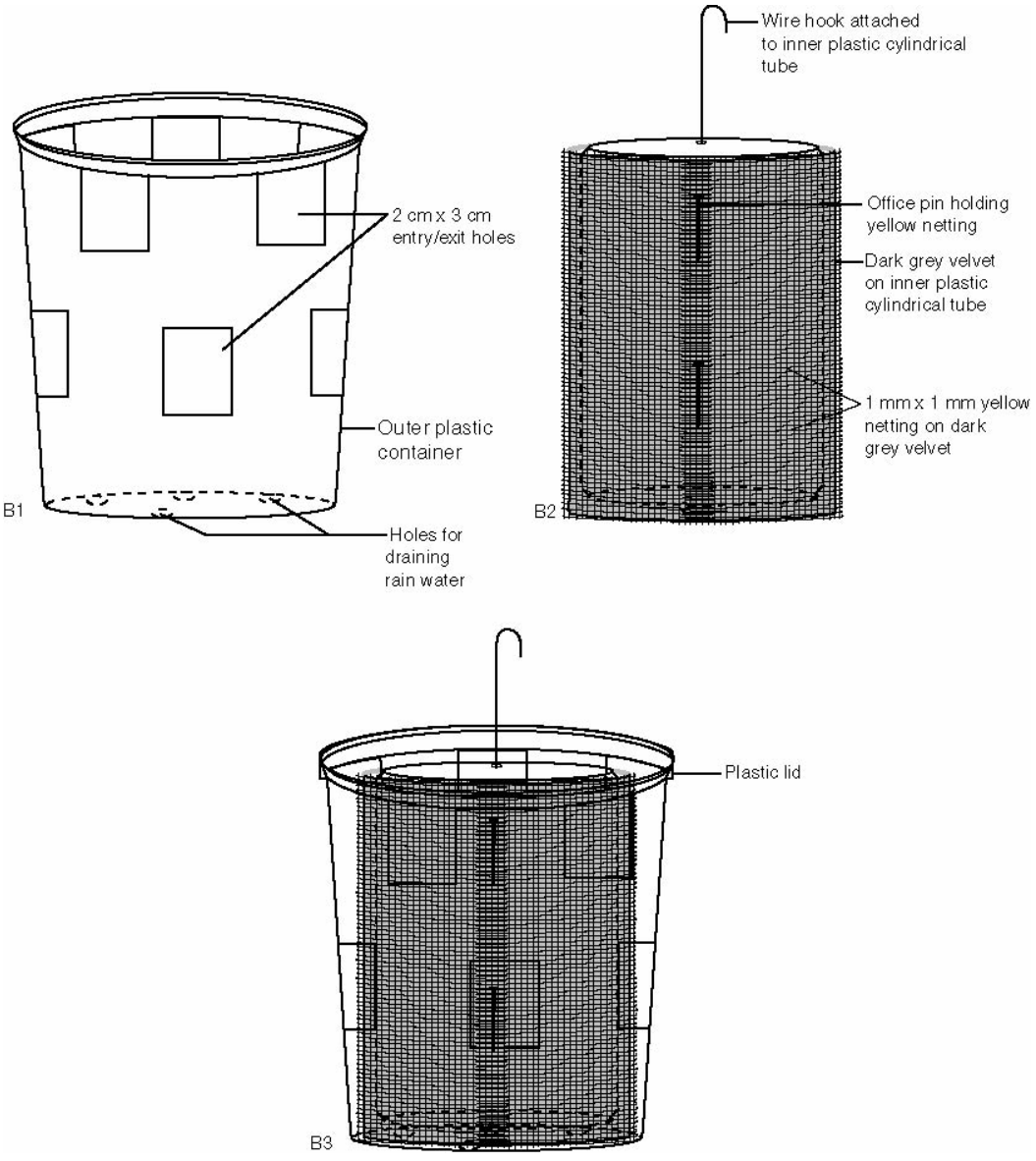

Figure 2.1: Autoinoculation device made from modified Lynfield trap (device B).

2.4.2.1 Evaluation of autoinoculation device in field cage

The mean number of spores picked up by a single fly visiting device B increased steadily ($F = 56.78$; $df = 4, 36$; $P < 0.0001$) with days after inoculation. One day after exposure, individual adult flies picked-up an average of 4.1×10^5 conidia in contrast to 39.6×10^5 conidia five days after inoculation (Table 2.3). Mortality of the flies in the control cages ranged between 0 and 35% (Table 2.3). Mortality of flies collected from the fungus-treated cage on two-five sampling days was significantly different from one-day sampling ($F = 19.46$; $df = 4,15$; $P < 0.0001$; Table 2.3). The LT_{50} values varied among the sampling dates ($F = 8.85$; $df = 4,15$; $P < 0.0007$), the shortest (1.8 days) being at three days after exposure and the longest (3.4 days) at one day after exposure (Table 2.3). The cumulative mortality in the different sampling days increased progressively reaching 100% over six days following treatment (Figure 2.2). There was no significant difference between the number of pupae collected in the control (2976.0) and fungus treatments (2400.8) ($Z = -1.9$; $P = 0.1$).

Table 2.3 Mean percentage mortality (X ± SE) of control and fungal infected adult *Liriomyza huidobrensis* at four days post-exposure, LT_{50} values and mean number of conidia per single fly exposed to *Metarhizium anisopliae* isolate ICIPE 20.

Sampling day after exposure	% Mortality of control (X ± SE)	% Mortality of infected (X ± SE)	LT_{50} values (X ± SE)	Mean no. of conidia/fly x 10^5 (X ± SE)
1	0b	68.4 ± 4.9b	3.4 ± 0.1a	4.1 ± 0.7d
2	28.75± 2.39ab	100a	2.4 ± 0.2bc	12.3 ± 1.5c
3	30.00± 2.89ab	100a	1.8 ± 0.2c	25.1 ± 2.1b
4	25.00± 2.89ab	97.8 ± 2.2a	2.3 ± 0.2bc	32.3 ± 3.3b
5	35.00 ± 2.04a	98.2 ± 1.8a	2.6 ± 0.2b	39.6 ± 4.0a

Means within columns followed by same letter are not significantly different, ANOVA and Student-Newman-Keuls (SNK) comparisons of means, $\alpha = 0.05$, n = 20.

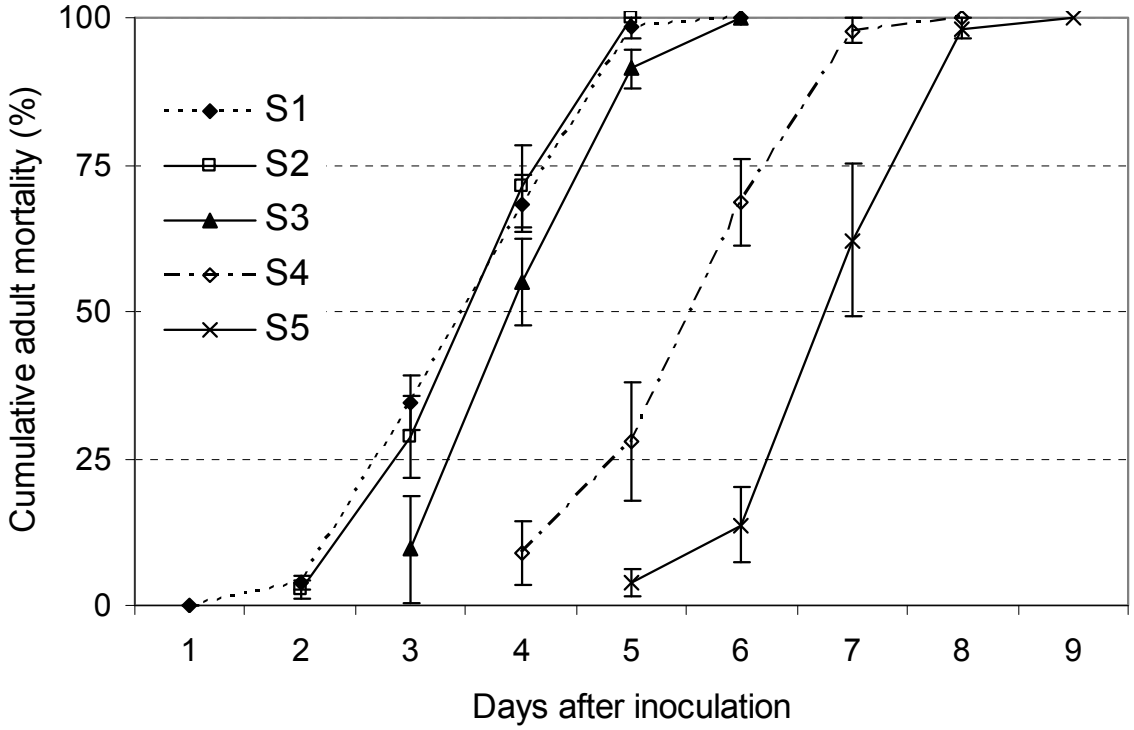

Figure 2.2 Infectivity of *Metarhizium anisopliae* applied in inoculation device showing cumulative mortality of adult *L. huidobrensis* over six days collected after different sampling days (S1-S5). n = 80.

2.5 Discussion

The potential of entomopathogenic fungi for control of insect pests has been demonstrated (Zimmermann, 1986; Ferron *et al*., 1991; Inglis *et al*., 2001). However, the success in the development of entomopathogenic fungi as mycoinsecticides involves several steps including isolation from the environment or diseased insects, strain selection based on several selection criteria such as efficacy and pathogen storage properties and formulation (Soper and Ward, 1981; Butt and Goettel, 2000). The results of laboratory screening have shown that all the 20 fungal isolates were pathogenic to adult *L. huidobrensis.* There was, however, considerable variation in the virulence among fungal isolates as

shown by mortality and lethal time values. The intra-specific variations in the pathogenic activity of entomopathogenic fungi observed in our study is similar to those reported for other arthropod pests (Ekesi *et al*., 1998; Dimbi *et al*., 2003; Quesada-Moraga *et al*., 2006; Bugeme *et al*., 2009) including leafminer immature stages (Borisov and Ushchekov, 1997; Bordat *et al*., 1988). In our study, none of the tested isolates originated from the host or closely related species, demonstrating that isolates of diverse origin can be equally pathogenic to *L. huidobrensis*. These results also demonstrate that some isolates of both *M. anisopliae* and *B. bassiana* are highly virulent to *L. huidobrensis*.

The number of conidia picked up by a single fly from the contamination tube ranged between 4.4 x 10^4 and 1.6 x 10^5. Using a similar infection technique, Dimbi *et al*. (2003) obtained between 4.2 x 10^5 and 1.0 x 10^6 conidia per single fruit fly. This difference could be attributed to the amount of conidia (0.1g) used in our study compared to 0.3g of conidia used by Dimbi *et al*. (2003), and the size of fruit flies, which are bigger than leafminer flies. The percent mortality was positively correlated with the amount of conidia picked up by a single fly from the contamination tube, suggesting that fungal infection of leafminers will depend on, among other factors, the pathogen properties such as ability to adhere to the host cuticle and the type of formulation.

Autodissemination devices have been developed, whereby insects are used to vector inoculum among conspecifics in the environment after they have been attracted and acquired the pathogen (Vega *et al*., 2007). This technique has been successfully tested against tsetse flies (Maniania *et al*., 2006) and fruit flies (Ekesi *et al*., 2007). Subsequently, for this study, an autoinoculation device was developed from a modified Lynfield trap and tested in field cage experiments. Based on this study, leafminer flies were attracted to the device and were able to pick up a lethal dose of inoculum (4.1 x 10^5 to 4.0 x 10^6 conidia per fly), resulting in higher adult mortality. Flies exhibited different behaviors inside the traps: (1) some flies landed on the device and almost exited immediately; (2) others walked

on the sides of the device before exiting; (3) some entered the trap and walked on the inner side of the trap before exiting; and (4) others were seen jumping up and down and either wriggled or groomed before exiting. This corroborates with the observations by Maniania (2002) who reported that insect behavior could affect the amount of inoculum picked up by a single fly in the contamination device.

The performance of the selected autoinoculation device was investigated in terms of delivering conidia into fly populations in field cages. All flies sampled at different time intervals (1-5 days after introduction of the treated device) succumbed to fungal infection with development of mycosis caused by *M. anisopliae,* which is an indication of successful contamination of flies through the device. However, our results still do not address the issue of fly-to-fly transmission, which is fundamental to the success of this technique. Further studies will be required to demonstrate whether a proportion of fungus-infected flies could transfer infection to the healthy ones. The viability of spores in the device over time was also not studied. However, Maniania (1998) reported that the pathogenic activity of *M. anisopliae* conidia to tsetse flies remained unchanged at eight days post exposure and could retain their infectivity for more than 21 days when applied to an infection chamber under field conditions. The device made is cost effective since materials used are locally available and the device can be produced locally. Once produced, the device can be reused over a long period of time requiring only cleaning and replenishing of conidia.

The number of pupae collected from the control and fungus treatments were not significantly different in the current study. There is no clear explanation for these results but the fact that not all flies became infected at the same time and that adults do not die immediately following infection implies that flies still have time to lay eggs before dying. These results emphasize the need for timing fungal application before population build-up if full use of the pathogen is to be made. It

further emphasizes the need for integration of entomopathogenic fungi with other pest control strategies for the management of leafminers.

The results of our study suggest that *M. anisopliae* (ICIPE 315, 69, 78, 07, 60, 62, 84, 20, 387, 18) and *B. bassiana* (ICIPE 273, 603, 279) could be considered for further development as microbial control agents of the pea leafminer while the contamination device could further be evaluated for field application of fungus for adult leafminer management.

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CHAPTER THREE

3. 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

3.1 Abstract

The effect of fungal infection by *Metarhizium anisopliae* on feeding and oviposition of adult *Liriomyza huidobrensis* was examined on three host plants, faba bean (*Vicia faba*), French bean (*Phaseolus vulgaris*) and snow pea (*Pisum sativum*) in the laboratory. Flies were contaminated with dry conidia through a velvet material wrapped around the inner side of a cylindrical plastic tube and allowed to feed and oviposit on the different host plants. A lactophenol and acid fuchsin solution was then used to stain leaves in order to count the number of eggs laid in the leaf tissues. The average number of punctures/cm^2 by *L. huidobrensis* in the three host plants generally increased from 48 h in the controls while it decreased from 72 h post-infection in *M. anisopliae* treatments. Generally, uninfected *L. huidobrensis* from the control treatment made more punctures in leaves than infected flies from 72 h post-treatment for the three host plants. The number of eggs increased over time in the control from 48 h post-treatment while it decreased from 96 h post-inoculation in fungus-treated female insects. Female *L. huidobrensis* laid more eggs when not infected than when they were infected by fungi. Host plant did not have any effect on feeding but had an influence on oviposition, with faba bean receiving a greater number of eggs in both the control and the *M. anisopliae* treatments. Our results show that infection by *M. anisopliae* can significantly reduce feeding and oviposition by *L. huidobrensis.* Furthermore, host plant affects leafminer oviposition and insects reared on faba bean were less susceptible to fungal infection than those reared on French bean and snow pea.

3.2 Introduction

The pea leafminer, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), is an economically important pest of a variety of flower and vegetable crops worldwide (Spencer, 1973; Weintraub and Horowitz, 1995; Wei *et al*., 2000; Martin *et al*., 2005; Mckee *et al*., 2009). It is an invasive species from the western United States and has been reported attacking plants in 14 plant families (Spencer, 1990; Wei *et al*., 2000; Martin *et al*., 2005; Mckee *et al*., 2009). The success of establishment of *L. huidobrensis* is attributed to its polyphagous nature, multi-voltinism, and the extreme capacity to develop resistance to different pesticides (Shepard *et al*., 1998; MacDonald, 1991).

Adults damage crops by puncturing the leaf surface to feed and to lay eggs into the leaf tissue (Parrella, 1987). When the eggs hatch, larvae tunnel within the leaf tissue forming damaging and disfiguring mines (Spencer, 1973; Knodel-Montz *et al*., 1985; Ameixa *et al*., 2007). Due to the widespread and significant damage that leafminers cause on various crops around the world, there is need to develop environmentally sound and sustainable methods of control for this pest. Biological control of *Liriomyza* leafminers has mainly focused on the use of parasitoids (Waterhouse and Norris, 1987; Johnson, 1993; Murphy and La Salle, 1999). The potential of entomopathogenic fungi as biological control agents for dipteran leafminers has recently been demonstrated (Bordat *et al*., 1988; Borisov and Ushchekov, 1997; Migiro *et al*., 2010). However, there is a lack of information about the extent to which entomopathogenic fungi may affect the feeding and fecundity of leafminers that develop on different host plants. It has been reported that fungal infection by mitosporic entomopathogenic fungi can affect food consumption and oviposition of insects (Fargues *et al*., 1991, 1994; Moore *et al*., 1992; Seyoum *et al*., 1994; Thomas *et al*., 1997; Ekesi and Maniania, 2000; Ondiaka *et al*., 2008). In addition, the virulence of the pathogen may vary according to the host plants or cultivars (Cory and Hoover, 2006).

The objectives of this study were therefore to evaluate the effects of the fungal pathogen *M. anisopliae* isolate ICIPE 20 on oviposition and feeding of *L. huidobrensis* on different host plants.

3.3 Materials and methods

3.3.1. Host plants

Three host plants namely faba bean, *Vicia faba* L., snow pea, *Pisum sativum* L. (cv. Oregon II) and French bean, *Phaseolus vulgaris* L. (cv. Samantha) (Fabales: Fabaceae) were used in the experiments. Plants were grown in a screen house (2.8 m length x 1.8 width × 2.2m height) in 15 cm pots (5-8 plants per pot), using a mixture of manure and soil in a ratio of 1:5.

3.3.2. Insects

Adult flies of *L. huidobrensis* were obtained from the Animal Rearing and Quarantine Unit, at the International Centre of Insect Physiology and Ecology (*icipe*). The colony had originally been reared for several generations on faba bean. Before use in the experiments, the leafminers were further reared for three generations on the three host plants mentioned above. They were maintained in a rearing room at 25-27°C, 60-80% R.H. and 12 L: 12 D photoperiod. Adults were fed on 10% sugar solution provided in balls of cotton wool soaked in the sugar solution and placed at the bottom corner of the rearing cages. In all the bioassays, one to two day-old naive adult flies were used.

3.3.3. Fungal isolate

Metarhizium anisopliae (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) isolate ICIPE 20 was used in this study. It has been reported earlier to be virulent to *L. huidobrensis* (Migiro *et al*., 2010). The isolate was cultured on Sabouraud Dextrose Agar in 9 cm Petri dishes and incubated at 25 ± 2°C in complete darkness. Conidia were harvested from three week-old cultures by scraping using a clean sterile spatula. The viability of conidia was then determined by spread-plating 0.1 ml of suspension titrated 3 x 10^6 conidia ml^{-1} onto 9 cm Petri

dishes containing SDA medium. A sterile microscope cover slip was placed on each plate and plates incubated in complete darkness at 25 ± 2°C and examined after 20 h. Percentage germination of conidia was determined by assessing whether a germ tube had formed in 100 random conidia on the surface area covered by each coverslip under the light microscope (400X). There were four replicate plates per isolate.

3.3.4. Inoculation of insects

Leaflets of 2-week old plants of the three host plants were cut at the stem and kept on their twigs (with each twig containing only four leaves). The foliage was then cleaned with a solution of sodium hypochlorite (2%) and rinsed thrice in sterile distilled water. For each host plant, two twigs were placed in a 20-ml universal bottle containing tap water to prevent the twigs from drying. The bottles were then plugged with cotton wool to prevent flies from entering into bottles and drowning as well as to maintain humidity near saturation inside bottles. Each bottle was then transferred into a clean ventilated Perspex cage (150 x 150 x 200 mm). Flies were inoculated using the technique described by Migiro *et al*. (2010). Briefly, flies were exposed to 0.1g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (70 mm length × 48 mm diam). For each host plant, 20 flies (10 males and 10 females) were transferred into the cylindrical tube and allowed to walk on the velvet for one minute, after which only five of the male and female insects were transferred from the velvet into the cages containing the different host plants. Insects in the control treatment were exposed to fungus-free velvet cloth before being transferred into similar cages. The treatments were maintained at 25 ± 2°C, 60-70% RH and 12L: 12D photoperiod. The experiment was replicated five times with 10 flies (five males + five females)/host plant/replicate. Test insects were provided with fresh foliage on twigs after 24, 48, 72, 96 and 120 h post-inoculation.

Leaves containing eggs and punctures were cut off the twigs daily, transferred into Petri dishes which were sealed with parafilm, placed in a plastic bag and

stored in a fridge at 4°C until the termination of the experiment. The number of feeding punctures and eggs was recorded using a modified egg staining technique described by Simonet and Pienkowski (1977) for potato leafhopper in alfalfa. Five hundred milliliters of lactophenol and acid fuchsin solution was prepared by mixing 100 ml distilled water, 100 ml lactic acid, 200 ml glycerin, 100 ml melted phenol crystals and 0.5g of acid fuchsin stain. The solution was heated to 95°C, after which leaves were dipped into the boiling solution for 5 minutes. The stained leaves were then transferred into 9 cm glass Petri dishes and left overnight. To remove excess stain, leaves were rinsed four times with warm water. Eggs and punctures on the entire top and bottom leaf surfaces for different time intervals were counted using a dissecting microscope at 85X magnification. Since leaf size varied with host plant, leaf area was converted in unit area for comparison purpose. Forty leaves per host plant were randomly selected and scanned using Adobe Photoshop, after which the leaf area in pixels was measured and then converted to surface area (cm^2). The number of eggs and punctures per surface area was then calculated.

3.3.5. Statistical analysis

Data on number of punctures and eggs/cm^2 among the different times post-infection for the three host plants and overall number of punctures and eggs/cm^2 among host plants were analysed using PROC GLIMMIX with a normal error distribution and an identity link function. Following a significant F-test, means were separated by the macro PDmix 800 for SAS (Saxton, 1998) using the Tukey-Kramer comparison procedure (α = 0.05) (SAS version 9.2; SAS Institute, Cary, NC). The Wilcoxon-Mann-Whitney U-test (SAS version 9.2; SAS Institute, Cary, NC) was used to compare the number of eggs and punctures cm^{-2} between the infected and uninfected *L. huidobrensis* treatments.

3.4 Results

3.4.1 Effect of fungal infection on feeding of *L. huidobrensis* on different host plants

The overall mean number of punctures did not differ significantly among host plants in both the control (fungus-free) ($F = 0.11$, $df = 2,117$; $P = 0.8922$) and fungus-treated ($F = 0.26$; $df = 2,117$; $P = 0.7688$) treatments (Table 3.1). However, there were significant differences in the mean number of punctures/cm^2 among the different times post-inoculation for the control treatments: faba bean ($F = 5.53$; $df = 4,195$; $P = 0.0003$), French bean ($F = 4.11$; $df = 4,195$; $P = 0.0032$) and snow pea ($F = 7.49$; $df = 4,195$; $P < 0.0001$), and for the fungus treatments: faba bean ($F = 21.79$; $df = 4,195$; $P < 0.0001$), French bean ($F = 14.88$; $df = 3,156$; $P < 0.0001$) and snow pea ($F = 37.97$; $df = 3,156$; $P < 0.0001$) (Table 3.2). For the controls, the number of punctures on faba bean and French bean significantly increased from 24 h post-inoculation and remained similar in subsequent times. However, for snow pea, it increased from 24 to 72 h and declined from 96 h post-inoculation (Table 3.2). For the fungus-inoculated treatments, the number of punctures generally, increased from 24 to 48 h post-inoculation and declined thereafter in all three host plants (Table 3.2). Compared to the control treatments, fungal treatments significantly reduced the number of punctures at 72, 96 and 120 h post-inoculation in faba bean, at 72 and 96 h post-infection in French bean, and at 24, 72, 96 h post treatment in snow pea (Table 3.2).

Table 3.1 Overall mean number of punctures produced by *Liriomyza huidobrensis* following infection with *Metarhizium anisopliae* on three host plants (at 120 h post-inoculation).

Host plant	Control	*Metarhizium anisopliae*
Faba bean	47.27 ± 3.13a	26.87 ± 2.28a
French bean	50.15 ± 3.60a	23.08 ± 1.88a
Snow pea	52.58 ± 4.27a	23.48 ± 3.14a

Within columns, means followed by the same letters are not significantly different (Tukey-Kramer, α = 0.05).

Table 3.2 Mean number of punctures (cm^{-2} leaf area) produced by *Liriomyza huidobrensis* following infection with *Metarhizium anisopliae* on three host plants among different times post-inoculation.

Host plant/time post-inoculation (h)	Number of punctures cm^{-2}		Statistical tests	
	Control	*Metarhizium anisopliae*	Z	P
Faba bean				
24	5.12 ± 1.11b	4.78 ± 0.94bc	0.71	0.4786
48	12.44 ± 1.25a	12.03 ± 1.37a	- 0.58	0.5603
72	11.02 ± 1.32a	7.21 ± 0.90ab	- 1.96	0.0496
96	9.51 ± 1.48ab	2.85 ± 1.02c	- 3.29	0.0010
120	9.19 ± 1.05ab	1.11 ± 0.52d	- 6.56	0.0001
French bean				
24	6.48 ± 1.02b	5.84 ± 1.06ab	-0.89	0.3733
48	13.37 ± 1.40a	10.32 ± 1.33a	-1.65	0.0989
72	13.97 ± 1.35a	4.88 ± 0.73b	-5.06	0.0001
96	9.01 ± 1.11ab	2.03 ± 0.70c	-5.75	0.0001
120	7.32 ± 1.21ab	-	-	-
Snow pea				
24	7.50 ± 1.63b	3.89 ± 1.09b	-3.15	0.0017
48	18.08 ± 2.38a	13.16 ± 2.25a	-1.62	0.1061
72	15.28 ± 1.97a	6.28 ± 1.26b	-3.50	0.0005
96	5.95 ± 1.17b	0.15 ± 0.10 c	-5.82	0.0001
120	5.77 ± 1.52b	-	-	-

Within columns, means followed by the same letters are not significantly different (Tukey-Kramer, α = 0.05); Wilcoxon-Mann-Whitney U-test (Z) used for comparisons across rows.

3.4.2 Effect of fungal infection on oviposition by *L. huidobrensis* on different host plants

The overall mean number of eggs among the three host plants in both the control (fungus-free) ($F = 20.40$; $df = 2,117$; $P < 0.0001$) and fungus-inoculated ($F = 18.75$; $df = 2,117$; $P < 0.0001$) treatments differed significantly (Table 3.3). Female *L. huidobrensis* in the control and fungus treatments laid more eggs on faba bean than French bean and snow pea (Table 3.3). The mean number of eggs in the control treatments differed significantly among the different times post-inoculation for faba bean ($F = 33.45$; $df = 4,195$; $P < 0.0001$), French bean ($F = 20.53$; $df = 4,195$; $P < 0.0001$) and snow pea ($F = 7.37$; $df = 4,195$; $P < 0.0001$) (Table 3.4). Generally, an increase in the mean number of eggs was observed from 24 to 48 h post-inoculation, after which the number remained similar (Table 3.4). Significant differences in the mean number of eggs laid by fungus-infected *L. huidobrensis* among the different times post-infection were also observed for faba bean ($F = 27.09$; $df = 4,195$; $P < 0.0001$), French bean ($F = 27.97$; $df = 3,156$; $P < 0.0001$) and snow pea ($F = 9.92$; $df = 3,156$; $P < 0.0001$) (Table 3.4). There was an increase in the mean number of eggs from 24 to 72 h post-infection followed by a decline in the subsequent times post-treatment for the three host plants. No eggs were recorded on French bean and snow pea at 120 h post-inoculation since all treated insects had died by that time (Table 3.4). Infection by *M. anisopliae* significantly reduced the number of eggs in faba bean and French bean across the different times post-infection. In snow pea, however, reduction in the number of eggs was recorded only at 96 h post-infection (Table 3.4).

Table 3.3 Overall mean number of eggs laid by *Liriomyza huidobrensis* following infection with *Metarhizium anisopliae* on three host plants (at 120 h post-inoculation).

Host plant	Control	*Metarhizium anisopliae*
Faba bean	6.09 ± 0.33a	1.48 ± 0.13a
French bean	1.29 ± 0.11b	0.57 ± 0.18b
Snow pea	0.59 ± 0.12c	0.24 ± 0.04c

Within columns, means followed by the same letters are not significantly different (Tukey-Kramer, α = 0.05).

Table 3.4 Mean number of eggs (cm^{-2} leaf area) produced by *Liriomyza huidobrensis* following infection with *Metarhizium anisopliae* on three host plants among different times post-inoculation.

Host plant/time post-inoculation (h)	Number of eggs cm^{-2}		Statistical tests	
	Control	*Metarhizium anisopliae*	Z	P
Faba bean				
24	0.11 ± 0.02b	0.06 ± 0.02c	-2.41	0.0092
48	1.15 ± 0.11a	0.48 ± 0.06 ab	-4.58	0.0001
72	1.56 ± 0.18a	0.52 ± 0.08 a	-4.78	0.0001
96	1.54 ± 0.18a	0.43 ± 0.06ab	-5.21	0.0001
120	1.73 ± 0.16a	0.25 ± .06b	-6.99	0.0001
French bean				
24	0.07 ± 0.01b	0.02 ± 0.01c	-2.46	0.0138
48	0.31 ± 0.04 a	0.15 ± 0.04ab	-3.15	0.0016
72	0.34 ± 0.06a	0.29 ± 0.17a	-3.83	0.0001
96	0.30 ± 0.05a	0.11 ± 0.03b	-3.82	0.0001
120	0.27 ± 0.06a	-	-	-
Snow pea				
24	0.06 ± 0.02c	0.06 ± 0.02ab	0.41	0.6800
48	0.09 ± 0.03bc	0.05 ± 0.02bc	-0.93	0.3543
72	0.10 ± 0.02bc	0.10 ± 0.03a	-1.10	0.2712
96	0.13 ± 0.05ab	0.03 ± 0.01c	-2.16	0.0312
120	0.21 ± 0.11a	-	-	-

Within columns, means followed by the same letters are not significantly different (Tukey-Kramer, α = 0.05); Wilcoxon-Mann-Whitney U-test (Z) used for comparisons across rows.

3.5 Discussion

In both the control and fungus treatments, the number of punctures made by flies increased from 24 to 48 h post-inoculation for the three host plants. However, thereafter, they remained similar in the controls while they decreased in fungus treatments. These results indicate the debilitating effect of *M. anisopliae* infection on feeding behavior of adult leafminers. The lag of time between inoculation and the onset of the fungus infection of flies, as expressed by the decrease in feeding may be explained by the fact that entomopathogenic fungi are slow-killing agents. Reduction in food consumption has been reported in a number of insects as fungal infection develops (Moore *et al*., 1992; Ekesi and Maniania, 2000; Ondiaka *et al*., 2008). Tyrrell (1990) attributed reduction in feeding of *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae) and *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae) larvae following infection with *Entomophaga aulicae* (Reichardt in Bail) Humber to the production of hyphal bodies, which invaded vital organs, thereby disrupting their functioning. Since leaf punctures are also used for oviposition (Bethke and Parrella, 1985), any reduction in the number of punctures will result in reduced damage and consequently a reduction in fecundity.

In the present study, host plants did not have significant effect on the number of punctures produced by either the uninfected or fungus-infected *L. huidobrensis*. This observation is in disagreement with Videla *et al*. (2006) who reported that *L. huidobrensis* caused more punctures on faba bean compared to *P. vulgaris* in the field. Yet in another study, Wei *et al*. (2000) reported that in a choice-test set-up, *L. huidobrensis* made more punctures on *V. faba* than on *P. sativum*, whereas in a no-choice set-up, a similar number of feeding punctures were made on the two host plants. The later finding is in agreement with our results since our experiment was also conducted in a no-choice experiment.

Metarhizium anisopliae-infected flies laid fewer eggs than the control flies. Similar results on reduced oviposition were reported by Blanford and Thomas (2001) in

desert locust, *Schistocerca gregaria* Forskål, infected with *Metarhizium anisopliae* var. *acridum*, Ekesi and Maniania (2000) in legume flower thrips, *Megalurothrips sjostedti* (Trybom) infected with *M. anisopliae* and Ondiaka *et al*. (2008) in sweetpotato weevil, *Cylas puncticollis* Boheman infected with *M. anisopliae*.

Female *L. huidobrensis* were still able to lay some eggs at the earlier stage of infection (72 h post-inoculation) before a decline started to occur which coincided with the reduction in the number of punctures by *L. huidobrensis*. Host plant had a significant effect on oviposition with faba bean receiving the greater number of eggs in both the control and fungus-infected treatments. Faba bean is considered as the most suitable host plant for rearing *L. huidobrensis* since insects reared on this plant are larger and yield high biomass exploitation (Salvo and Valladares, 2002; Martin *et al*., 2005). Host plants may have an influence on host insect-pathogen relationships such as influencing pathogen acquisition and infection rates (Cory and Hoover, 2006). Phytochemicals, especially allelochemicals and nutrients can modify the physiology and growth of the insect host as well as the pathogen, thereby affecting its susceptibility to infection (Ali *et al*., 1998; Ekesi *et al*., 2000; Cory and Hoover, 2006). Differences in nutritional quality, plant chemistry, leaf topography and insect behaviour might have acted in combination or independently in affecting the efficacy of *M. anisopliae* on feeding and oviposition of flies on the different host plants used in the study. Fungus-infected flies on faba bean were able to survive longer, feed and lay eggs up to 120 h post-inoculation, suggesting that they were most fit and could withstand infection for longer periods compared to those on French bean and snow pea. This work was, however, not designed to investigate the effect of sequestered antimycotic phytochemicals on *L. huidobrensis* performance and susceptibility to infection.

In conclusion, our study showed that, infection by *M. anisopliae* can significantly reduce feeding and oviposition by *L. huidobrensis*. However, since reductions in punctures and eggs generally occur after 72 h post-inoculation, there is the need

for integrated approach and timely application of the fungus before onset of peak feeding and oviposition in leafminers. A better understanding of the interaction between the insect, host plant and the pathogen is important since factors such as behavioural, physiological and biochemical may act in combination or independently resulting in differences in efficacy of *M. anisopliae* on insects feeding on different host plants.

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CHAPTER FOUR

4. Effect of constant temperatures and host plant on the virulence of *Metarhizium anisopliae* isolates to three species of adult leafminers, *Liriomyza huidobrensis*, *Liriomyza trifolii* and *Liriomyza sativae*

4.1 Abstract

The effect of constant temperatures (15°C, 20°C, 25°C, 28°C) on the virulence of five fungal isolates of *Metarhizium anisopliae* against three species of leafminers, *Liriomyza huidobrensis, L. sativae* and *L. trifolii* was studied in the laboratory. The effect of two host plants, French bean, (*Phaseolus vulgaris*) and faba bean (*Vicia faba*) on the susceptibility of the three leafminer species to one virulent *M. anisopliae* isolate, ICIPE 20 was also investigated. Insect mortality varied with temperature, fungal isolate and leafminer species. Fungal isolates were more virulent at 25°C and 28°C than at 15°C and 20°C. The lethal time to 50% mortality (LT_{50}) values decreased with increasing temperature. Leafminer mortality was affected by both the concentrations used (1 x 10^5, 10^6, 10^7 conidia ml^{-1}) and the host plant. Mortality was higher on French bean than on faba bean at all the concentrations for the three leafminer species. The lethal time to 50% mortality (LT_{50}) values were higher on faba bean than on French bean for the three leafminer species. The lethal concentration (LC_{50}) was only higher for *L. huidobrensis* reared on faba bean compared to those reared on French bean. For both *L. sativae* and *L. trifolii*, however, the concentrations did not differ significantly between the two host plants.

4.2 Introduction

The *Liriomyza* leafminers, *Liriomyza huidobrensis, L. sativae* and *L. trifolii* are invasive to Africa and are polyphagous, causing considerable economic damage

to several vegetable and ornamental plants worldwide (Spencer, 1973; Murphy and Lasalle, 1999). In Kenya, they have been reported attacking several vegetable and ornamental crops such as snow peas (*Pisum sativum* L. var. *saccharatum*), courgettes (*Cucurbita pepo* L.) (Cucurbitales: Cucurbitaceae)), okra (*Abelmoschus esculentus* (L.) Moench) (Malvales: Malvaceae)), onions (*Allium cepa* L.) (Asparagales: Alliaceae)), beans (*Phaseolus vulgaris* L.) (Fabales: Fabaceae)), gypsophila (*Gypsophila* sp.) (Caryophyllales: Caryophyllaceae)) and chrysanthemums (Chrysanthemum sp.) (Asterales: Asteraceae)) (Kabira, 1985; Spencer, 1985; KEPHIS, 2009; ICIPE, unpublished). In addition to the damage they cause, these leafminers are quarantine pests and their presence on export produce can lead to rejection, resulting in loss of export markets and consequently loss of revenue. For instance, between July 2008-June 2009 alone, a total of 16 interceptions due to leafminer damage in vegetable produce were reported from the European market (KEPHIS, 2009). The use of chemical pesticides in leafminer management has proven uneconomical mostly due to the rapid development of resistance among fly populations in addition to elimination of their natural enemies and the adverse effects to the environment (MacDonald, 1991; Weintraub and Horowitz, 1995; Murphy and LaSalle, 1999). Entomopathogenic fungi are currently under investigation at the International Center of Insect Physiology and Ecology (*icipe*) for use in leafminer management. However, the efficacy of entomopathogenic fungi is affected by many biotic and abiotic factors. Temperature is one of the most important abiotic factors that affect the host, fungal growth and the interactions between the host and pathogen (Benz, 1987; Thomas and Jenkins, 1997). For instance, lower temperatures have been shown to retard the developmental cycle of some insect hosts thereby giving advantage to the pathogen to infect the host (Fargues and Remaudière, 1977). On the other hand, high temperature can lead to the shortening of the intermolt period in insects thereby preventing penetration of hyphae into the insect haemocoel (Fargues, 1972). Several studies have suggested that fungi tend to kill most rapidly at the optimum temperature for vegetative growth and germination (Doberski, 1981;

Lecuona and Alves, 1988; Moorehouse *et al*., 1994). Since temperature can affect germination, growth, survival and pathogenicity of the entomopathogenic fungi, it is important to investigate the effect of temperature on the pathogen and the host in order to select optimum strains for their development as microbial control agents.

Host plant is also one of the biotic factors that can affect the virulence of a fungal pathogen against a host, either through insect's diet, physical properties of the plant or direct antimicrobial activity of the plant (Tanada and Kaya, 1993; Cory and Hoover, 2006). Many plants produce antimicrobial compounds, which may inhibit the activity of entomopathogens (Gallardo *et al*., 1990; Lacey and Mercadier, 1998; Costa and Gaugler, 1989; Poprawski *et al*., 2000; Vega *et al*., 1997; Lacey and Mercadier, 1998; Inyang *et al*., 1998a). For instance, tannins, as secondary plant metabolites, can strongly inhibit growth and development of entomopathogens (Keating and Yendol, 1987; Inyang *et al*., 1998b). Also, alpha-tomatine has been shown to inhibit *in vitro* colony formation and growth of *Nomuraea rileyi* (Farlow) Samson (Gallardo *et al*., 1990). Antifungal compounds in leaf exudates or leaf volatiles may come into direct contact with conidia of entomopathogenic fungi and inhibit the germination and infection process (Ugine *et al*., 2007). For instance, Vega *et al*. (1997) showed that the presence of allelochemicals (catechol and salicylic acid) either on the leaf surface or insect cuticle had an inhibitory effect on the germination rate of the fungal pathogen *Isaria fumosorosea* (=*Paecilomyces fumosoroseus*) (Wize) Brown and Smith.

Insect herbivores are also known to sequester antimycotic phytochemicals that help confer resistance to fungal infection (Ramoska and Todd, 1985; Gallardo *et al*., 1990; Poprawski *et al*., 2000). Poprawski *et al*. (2000) found that third-instar nymphs of *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae) sequestered the glycoalkaloid tomatine making them less susceptible to infection. In another study, however, Storey *et al*. (1991) reported that the presence in the egg of a pyrrolizidiru alkaloid sequestered by an arctiid moth did

not protect it against *B. bassiana* or *Paecilomyces lilacinus* (Thom) Samson and had no effects on germination of the fungal pathogens.

The objectives of this study were therefore to i) investigate the effect of constant temperatures on the virulence of five isolates of *M. anisopliae* against three species of leafminers and ii) evaluate the effect of host plant on the susceptibility of three leafminer species to *M. anisopliae*.

4.3 Materials and methods

4.3.1 Host plants

Two host plants, the faba bean, *Vicia faba* L. and the French bean, *Phaseolus vulgaris* L. (cv. Samantha) (Fabales: Fabaceae) were used in the experiments. Plants were grown in screen houses (2.8 m length x 1.8 width × 2.2 m height) in 15 cm pots (5-8 plants per pot), using a mixture of manure and soil in a ratio of 1:5 at *icipe*'s Headquarters, Duduville, Nairobi, Kenya.

4.3.2 Insects

Adult flies of the three leafminer species (*L. huidobrensis*, *L. sativae* and *L. trifolii*) were obtained from the Animal Rearing and Quarantine Unit, *icipe*. The *L. huidobrensis* colony was maintained on faba bean while *L. sativae* and *L. trifolii* colonies were reared on French bean, for use in the temperature experiment. In the host plant bioassays, the three leafminer species were reared for a minimum of three generations on the two different host plants. All insects were maintained in a rearing room at 25-27°C, 60-80% RH and 12 L:12 D photoperiod. Adults were fed on 10% sugar solution (balls of cotton wool soaked in the sugar solution and placed at the bottom corner of the rearing cages). In all the bioassays, 1 to 2 day-old naïve adult flies were used.

4.3.3 Fungal isolates

Fungal isolates used in the study were obtained from the *icipe*'s Arthropod Germplasm Centre. These isolates were cultured on Sabouraud dextrose agar (SDA) in Petri dishes (9 cm) and incubated at 25 ± 2°C in complete darkness.

4.3.3.1 Preparation of the conidial suspension

Conidia were harvested from 3-week old cultures by scraping the surface using a sterile spatula. Spores were suspended in 20 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing 3 mm glass beads. The conidial suspension was vortexed for five minutes to produce a homogeneous conidial suspension. Conidia were then quantified using a hemocytometer following serial dilutions in sterile distilled water. Viability of the conidia was determined by spread-plating 0.1 ml of 3 x 10^6 conidia ml^{-1} onto 9 cm Petri dishes containing SDA medium. A sterile microscope cover slip (2 x 2 cm) was placed on top of the agar in each plate. Plates were incubated in complete darkness at 25 ± 2°C and examined after 20 hours. Percentage germination of conidia was determined from 100 random conidia on the surface area covered by each coverslip under the light microscope (400X) (Goettel and Inglis, 1997). Each plate represented a replicate with each isolate having four replicates.

4.3.4 Effect of temperature on virulence of *M. anisopliae* to leafminers

Adult flies were inoculated using the technique described by Migiro *et al*. (2010). Flies were exposed to 0.1g of dry conidia evenly spread on a cotton velvet cloth covering the inner side of a cylindrical plastic tube (70 mm length × 48 mm diam) at room temperature (25 ± 2°C). Flies were then transferred into clean ventilated Perspex cages (150 x 150 x 200 mm) and incubated at 15, 20, 25 and 28°C. Since preliminary bioassays at 30°C resulted in high mortalities in both the control and the fungal treatments, this temperature was excluded in the experiments. A ball of cotton wool soaked in 10% sugar solution was placed at the bottom corner of the cage as a food source. Insects in the control treatments were exposed to fungus-free velvet cloth before being transferred into similar

cages. The experiment was replicated four times with 20 insects per replicate. Mortality was recorded daily until all flies died. Dead insects were surface-sterilized in 70% alcohol followed by three rinses in sterile distilled water and transferred to Petri dishes lined with damp sterilized filter paper to allow fungal growth on the surface of the cadaver. Only mycosed flies were used for analysis.

4.3.5 Effect of host plant on the virulence of *M. anisopliae* to leafminers

Two-week old plants of the two host plants (faba bean and French bean) were cut at the stem and kept on their twigs. For each host plant, two twigs were placed in a 20 ml universal bottle containing tap water to prevent the twigs from drying. The bottles were then plugged with cotton wool to prevent flies from drowning and to maintain humidity near saturation in the bottles.

Leaves on twigs were sprayed directly with 10 ml of different fungal concentrations (1.0×10^5, 1.0×10^6 and 1.0×10^7 conidia ml^{-1}) of one of the virulent *M. anisopliae* isolate ICIPE 20 using a Burgerjon's (1956) spray tower. Controls were sprayed with sterile distilled water containing 0.05% Triton X-100. Each bottle containing the sprayed twigs with leaves was then transferred into a clean ventilated Perspex cage (150 x 150 x 200 mm).

Flies were then exposed to treated foliage from the host plant species on which they were reared. After 24 hours, treated foliage was replaced with fresh foliage which was changed on alternate days thereafter. The experiment was maintained at 25-27°C, 50-70% RH and 12L: 12D photoperiod. The experiment for each insect and host plant species was replicated 4 times with 20 flies/dose/replicate. Mortality was recorded daily until all the flies died. Dead flies were surface sterilized as described earlier and only mycosed flies were used in the analysis.

4.3.6 Statistical analysis

Percentage mortality data were corrected for control mortality (Abbot, 1925) and normalized by arcsine transformation (Sokal & Rohlf, 1981) before being

subjected to a three-way analysis of variance (ANOVA) at 95% level of significance. Student-Newman-Keul's (SNK) procedure was used to separate the means as a post ANOVA procedure (α = 0.05). The lethal time to 50% (LT_{50}) mortality (per replicate) and the medial lethal concentration (LC_{50}) values were determined using the probit analysis procedure for correlation data (Throne *et al*., 1995). The LT_{50} estimated values for each replicate were then compared among themselves using ANOVA and means separated using the Student-Newman-Keul's (SNK) (for the temperature experiment) and Least Significant Difference (LSD) (for the host plant experiment) tests. Chi-square and T-tests were used for comparisons between host plants for the different doses and leafminer species and for comparisons for LC_{50} and LT_{50} values between host plants for the three leafminer species respectively. These analyses were carried out using the SAS version 9.2 statistical package (SAS Institute, 2003).

4.4 Results

4.4.1 Effect of temperature on the virulence of *M. anisopliae* to leafminers

Mortality in the control treatments did not exceed 18% for all the isolates at all temperature regimes. Mortality of the adult leafminer flies was significantly affected by temperature (F = 589.33; df = 3,204; P < 0.0001), isolate (F = 27.86; df = 4,204; P < 0.0001) and leafminer species (F = 10.02; df = 2,204; P < 0.0001). Significant interactions between temperature and isolate (F = 5.74; df = 12,204; P < 0.0001) as well as isolate and leafminer species (F = 5.18; df = 8,204; P < 0.0001) were recorded. However, no interaction was observed between temperature and leafminer species (F =1.35; df = 6,204; P = 0.2347).

Fungal isolates differed significantly among the different temperatures in their virulence against the three leafminer species and were more effective at 25 and 28°C than at 15 and 20°C (Table 4.1). For example, for *L. huidobrensis*, isolate

ICIPE 18, the lowest mortality of 23.59 ± 8.0 and 32.11 ± 4.39 was recorded at temperatures 15°C and 20°C respectively, while the highest mortality of 80.12 ± 5.71 and 100 was recorded at temperatures 25°C and 28°C respectively ($F = 34.21$; $df = 3,12$; $P < 0.0001$). For *L. sativae*, ICIPE 18 recorded the lowest mortality of 40.99 ± 4.53 at 15°C followed by 69.65 ± 8.76 at 20°C, 88.61 ± 5.19 at 25°C and 100 at 28°C ($F = 23.27$; $df = 3,12$; $P < 0.0001$). For *L. trifolii*, ICIPE 18 recorded the lowest mortality of 61.97 ± 5.31 and 75.19 ± 4.63 at both 15°C and 20°C respectively, while the highest mortality of 79.02 ± 4.28 and 100 was recorded at 25°C and 28°C respectively ($F = 36.52$; $df = 3,12$; $P < 0.0001$). A similar trend was observed for all the other isolates and leafminer species (Table 4.1)

A comparison of the isolates for the three leafminer species and temperatures showed that, at 15°C, all isolates were equally virulent to *L. huidobrensis* while for both *L. sativae* and *L. trifolii*, isolate ICIPE 18 outperformed the other isolates. At 20°C, isolate ICIPE 84 was more virulent to *L. huidobrensis,* ICIPE 18 and 20 were more virulent to *L. sativae* while ICIPE 18 was more virulent to *L. trifolii*. At 25°C, all isolates were equally active for both *L. huidobrensis* and *L. sativae*. However, for *L. trifolii*, ICIPE 69 and 84 caused higher mortality compared to the other isolates. At 28°C, all isolates were equally active against both *L. huidobrensis* and *L. sativae*. For *L. trifolii*, however, all isolates were equally active except for ICIPE 20 which was the least active (Table 4.1).

The three leafminer species differed in their susceptibility to fungal infection at all temperatures, except at 28°C (Table 4.1). *Liriomyza huidobrensis* was less susceptible to isolate ICIPE 18 at both 15 and 20°C, ICIPE 20 at 20°C and ICIPE 78 at 25°C. *Liriomyza sativae* was less susceptible to isolate ICIPE 78 at 15°C and ICIPE 84 at 20°C while *L. trifolii* was less susceptible to isolate ICIPE 84 at 20°C (Table 4.1).

The lethal time to 50% mortality (LT_{50}) was significantly affected by temperature ($F = 671.76$; $df = 3,204$; $P < 0.0001$), isolate ($F = 27.12$; $df = 4,204$; $P < 0.0001$) and leafminer species ($F = 38.31$; $df = 2,204$; $P < 0.0001$). Significant temperature by isolate ($F = 11.54$; $df = 12,204$; $P < 0.0001$), temperature by leafminer species ($F = 22.25$; $df = 6,204$; $P < 0.0001$) and isolate by leafminer species ($F = 6.19$; $df = 8,204$; $P < 0.0001$) interactions were also recorded. The LT_{50} values decreased with increasing temperature and ranged from 3.1 to 12.6 days at 15°C, 2.6 to 6.4 at 20°C, 1.9 to 2.7 at 25°C and 1.2 to 2.1 days at 28°C (Table 4.2). The LT_{50} values differed significantly among fungal isolates for the three leafminer species at all temperature levels tested, except for *L. huidobrensis* at 28°C (Table 4.2).

Table 4.1 Percent mortality caused by five *Metarhizium anisopliae* isolates to three *Liriomyza* species three days after exposure to four temperature levels.

Host species	ICIPE 18	ICIPE 20	ICIPE 69	ICIPE 78	ICIPE 84
			15°C		
L. huidobrensis	23.59 ± 8.0bA	5.07 ± 0.07aA	7.50 ± 2.50aA	8.82 ± 2.36aA	15.46 ± 3.67aA
L. sativae	40.99 ± 4.53abA	21.08 ± 0.45aB	18.46 ± 4.38aB	0bC	19.01 ± 6.60aB
L. trifolii	61.97 ± 5.31aA	14.08 ± 6.24aB	20.26 ± 6.02aB	8.75 ± 4.27aB	24.14 ± 2.74aB
			20°C		
L. huidobrensis	32.11 ± 4.39bB	20.94 ± 4.19bBC	30.65 ± 6.03aB	10.07 ± 1.99aC	67.43 ± 8.30aA
L. sativae	69.65 ± 8.76aA	57.06 ± 9.66aA	26.97 ± 8.35aB	5.27 ± 0.11aC	26.61 ± 1.75bB
L. trifolii	75.19 ± 4.63aA	31.25 ± 10.80abB	18.88 ± 3.65aB	19.28 ± 7.53aB	37.93 ± 3.88bB
			25°C		
L. huidobrensis	80.12 ± 5.71aA	78.29 ± 9.51aA	79.46 ± 7.97aA	66.37 ± 5.02bA	94.66 ± 3.72aA
L. sativae	88.61 ± 5.19aA	93.27 ± 2.60aA	87.50 ± 4.74aA	83.77 ± 2.28aA	94.66 ± 2.15aA
L. trifolii	79.02 ± 4.28aB	84.06 ± 4.17aB	100aA	87.48 ± 1.41aB	98.53 ± 1.47aA
			28°C		
L. huidobrensis	100aA	93.33 ± 4.71aA	86.53 ± 8.33aA	95.27 ± 1.60aA	100aA
L. sativae	100aA	100aA	100aA	98.44 ± 1.56aA	100aA
L. trifolii	100aA	93.27 ± 1.27a B	100aA	95.40 ± 2.98aAB	100aA

Means (± SE) within column followed by the same lower case letter and within row followed by the same upper case letter are not significantly different by SNK ($P < 0.05$).

* Column mean separation is at each temperature among leafminer species for each isolate. Row mean separations are for each temperature among isolates for each leafminer species.

Table 4.2 Mean (± SE) lethal time (days) to 50% mortality (LT_{50}) for three *Liriomyza* species treated with isolates of *Metarhizium anisopliae* at four constant temperatures.

Species	ICIPE 18	ICIPE 20	ICIPE 69	ICIPE 78	ICIPE 84
			15°C		
L. huidobrensis	7.37 ± 0.64aC	8.89 ± 0.48aBC	12.63 ± 1.15aA	10.05 ± 0.39aB	8.31 ± 0.50aBC
L. sativae	3.44 ± 0.13bD	4.22 ± 0.04bCD	7.39 ± 1.89aB	11.08 ± 0.78aA	5.92 ± 0.65bBC
L. trifolii	3.13 ± 0.17bB	4.83 ± 0.24bA	6.72 ± 1.40aA	5.43 ± 0.24bA	4.12 ± 0.26cAB
			20°C		
L. huidobrensis	3.54 ± 0.15aB	3.89 ± 0.10aB	3.50 ± 0.15bB	4.76 ± 0.48bA	2.60 ± 0.18cC
L. sativae	2.71 ± 0.09bC	2.90 ± 0.24bC	3.71 ± 0.27bB	6.37 ± 0.21aA	3.88 ± 0.12aB
L. trifolii	2.69 ± 0.08bC	3.19 ± 0.13bBC	5.08 ± 0.48aA	3.89 ± 0.30bB	3.27 ± 0.06bBC
			25°C		
L. huidobrensis	2.61 ± 0.05aA	2.55 ± 0.16aA	2.20 ± 0.08aB	2.69 ± 0.12aA	2.05 ± 0.12aB
L. sativae	2.01 ± 0.01bB	2.42 ± 0.18aA	1.96 ± 0.10aB	2.11 ± 0.05bB	2.01 ± 0.01aB
L. trifolii	2.58 ± 0.06aA	2.40 ± 0.05aB	1.92 ± 0.08aC	2.01 ± 0.02bC	1.95 ± 0.03aC
			28°C		
L. huidobrensis	1.83 ± 0.14aA	1.83 ± 0.10aA	1.91 ± 0.23aA	1.71 ± 0.11aA	1.93 ± 0.06aA
L. sativae	1.73 ± 0.12aAB	2.08 ± 0.04aA	1.53 ± 0.14aB	1.94 ± 0.03aA	1.72 ± 0.12aAB
L. trifolii	1.22 ± 0.10bB	1.90 ± 0.04aA	1.71 ± 0.15aA	1.96 ± 0.02aA	1.89 ± 0.07aA

Means (± SE) within column followed by the same lower case letter and within row followed by the same upper case letter are not significantly different by SNK ($P < 0.05$).

* Column mean separation is at each temperature.

4.4.2 Effect of host plant on the virulence of *M. anisopliae* to leafminers

The susceptibility of the three leafminer species to fungal infection was significantly affected by the type of host plant (F = 13.84; df = 1,346; P = 0.0002) and fungal concentration (F = 3.24; df = 2,346; P = 0.0402) (Table 4.3). Adult flies reared on faba bean were less susceptible to fungal infection compared to those reared on French bean at all the concentrations (Table 4.3). In general, mortality increased with increased concentrations of *M. anisopliae* with all host plants (Table 4.3). Significant differences in LC_{50} values were recorded among the leafminers exposed to fungus-treated French bean (F = 4.48; df = 2,9; P = 0.0447) and faba bean (F = 19.40; df = 2,9; P = 0.0005), with *L. huidobrensis* recording higher concentrations than *L. sativae* and *L. trifolii* in both cases (Table 4.4). A comparison of LC_{50} values between the two host plants for the three leafminer species showed that *L. huidobrensis* recorded higher values on faba bean while no differences were recorded for both *L. sativae L. trifolii* (Table 4.4). The LT_{50} values varied significantly for all leafminer species among the two host plant species (Table 4.4). They were longest on insects reared on faba bean in all the three species of leafminers (Table 4.4). The LT_{50} values also differed significantly (F = 11.21; df = 2,9; P = 0.0036) among the leafminer species for faba bean, with *L. huidobrensis* having the longest LT_{50} (5.84 ± 0.42 days) compared to both *L. sativae* (4.34 ± 0.22 days) and *L. trifolii* (4.22 ± 0.11 days) which were similar. On French bean however, no significant differences (F = 0.36; df = 2,9; P = 0.7048) in LT_{50} values among leafminer species were recorded (Table 4.4).

Table 4.3 Percentage mortality (mean ± SE) of adult *Liriomyza huidobrensis*, *L. sativae* and *L. trifolii* reared on different host plants following treatment with *Metarhizium anisopliae* isolate ICIPE 20.

Leafminer species	Host plant	Concentration (conidia ml^{-1})			Statistic		
		1×10^5	1×10^6	1×10^7	F	Df	P
L. huidobrensis	French bean	46.41 ± 3.88b	71.81 ± 6.82ab	80.56 ± 8.64a	4.76	2,9	0.0389
	Faba bean	27.75 ± 2.0b	41.58 ± 3.03a	47.06 ± 4.13a	10.18	2,9	0.0049
	Chi-square	5.33, P=0.0209	5.33, P=0.0209	5.01, P=0.0251			
L. sativae	French bean	77.04 ± 2.62b	85.70 ± 1.67b	97.14 ± 1.65a	17.54	2,9	0.0008
	Faba bean	46.49 ± 1.32b	73.20 ± 1.57a	76.31 ± 6.08a	12.42	2,9	0.0026
	Chi-square	5.40, P=0.0202	5.39, P=0.0202	4.86, P=0.0275			
L. trifolii	French bean	75.33 ± 1.61c	87.01 ± 1.23b	94.28 ± 2.27c	12.30	2,9	0.0027
	Faba bean	59.31 ± 4.39b	61.11 ± 2.27b	77.78 ± 2.27a	11.11	2,9	0.0037
	Chi-square	4.80, P=0.0284	5.67, P=0.0172	5.46, P=0.0194			

Means (± SE) within row followed by the same letter are not significantly different by SNK ($P < 0.05$). χ^2 test used for column comparisons between host plants for each leafminer species and concentration.

Table 4.4 Lethal concentration (LC_{50}) and lethal time (LT_{50}) values for *Liriomyza huidobrensis*, *L. sativae* and *L. trifolii* reared on different host plants following treatment with *Metarhizium anisopliae* isolate ICIPE 20.

LC_{50} (Mean ± SE)				
Leafminer species	Host plant		Statistic	
	French bean	Faba bean	t	P
L. huidobrensis	$13.3 \times 10^4 \pm 4.18 \times 10^4$a	$1.84 \times 10^8 \pm 1.44 \times 10^8$ a	4.22	0.0243
L. sativae	$1.18 \times 10^4 \pm 0.7 \times 10^4$b	$9.81 \times 10^4 \pm 2.10 \times 10^4$b	2.60	0.0804
L. trifolii	$0.8 \times 10^4 \pm 0.3 \times 10^4$b	$3.93 \times 10^4 \pm 2.77 \times 10^4$b	1.32	0.2783
LT_{50} (Mean ± SE)				
L. huidobrensis	3.56 ± 0.26a	5.84 ± 0.42a	3.71	0.0340
L. sativae	3.39 ± 0.09a	4.34 ± 0.22b	3.84	0.0311
L. trifolii	3.33 ± 0.16a	4.22 ± 0.11b	3.40	0.0425

Means (± SE) within column followed by the same lower case letter are not significantly different by LSD ($P < 0.05$). T-test used for row comparisons between host plants for each leafminer species.

4.5 Discussion

The five fungal isolates caused mortality in all three species of leafminers, but disease development and mortality varied with temperature, isolate and host species. The fungal isolates were most effective at 25 and 28°C, with mortality ranging from 66 to 100% being achieved within 3 days in all three species. Several fungal isolates have been reported to be more pathogenic to arthropod pests at the temperatures ranges of 25 to 35° C. Ekesi *et al*. (1999) reported 25 and 30°C as the optimal temperature for development of *M. anisopliae* and *B. bassiana* isolates in *Megalurothrips sjostedti* (Trybom) while Dimbi *et al*. (2004) reported 30° C for *M. anisopliae* in *Ceratitis capitata* (Weidemann), *C. fasciventris* (Bezzi) and *C. cosyra.* Bugeme *et al*. (2008; 2009) found that temperatures of 25, 30 and 35° C were optimal for infection of *Tetranychus evansi* (Baker and Pritchard) and *T. urticae* Koch by *M. anisopliae* and *B. bassiana*. The findings of this study also showed that disease development increased with increased temperature. The choice of a fungal isolate for development as mycoinsecticide should therefore take into account several criteria including its ability to withstand a range temperatures (since in a field situation, seasonal and diurnal variations are experienced) and its ability to infect the target pest (Fargues *et al*., 1997; Ekesi *et al*., 1999; Dimbi *et al*., 2004; Bugeme *et al*., 2008; 2009).

The virulence of the *M. anisopliae* varied according to the concentration, the host plant and the leafminer species. The role of host plant in mediating the susceptibility of insect pests to fungal infection has been demonstrated elsewhere (Poprawski *et al*., 2000; Duetting *et al*., 2003; Ugine *et al.,* 2007). In our study, leafminers reared on French bean were more susceptible to *M. anisopliae* infection compared to those on faba bean. Although there is no explanation for this differential susceptibility, differences in physical and chemical properties among host plants are known to influence host-pathogen relationships (Poprawski *et al*., 2000; Cory and Hoover, 2006; Ugine *et al.,* 2007). Among the mechanisms that might have contributed to the greater mortality and mycosis of

leafminers by *M. anisopliae* on French bean as compared to faba bean, could be the leaf surface topography which is rough and hairy in French bean and smooth in faba bean. The topography of French bean might have allowed a greater retention of conidia on the surface, thereby increasing the contact with leafminers and resulting in high mortality. The degree of leaf roughness has been shown to play an important role in retention and spreading of insecticide droplets (Chowdhury *et al*., 2005) as well as fungal suspensions (Ugine *et al*., 2007; Olleka *et al*., 2009) with retention increasing with increasing roughness. Ugine *et al*. (2007) reported that the rate of conidial acquisition by *Franklinella occidentalis* (Pergande) was dependent on the host plant on which they were exposed to *B. bassiana*. Those exposed on *P. vulgaris* acquired more conidia resulting in higher rates of mortality compared to those on *Impatiens wallerana* Hook.f.

Qualitative and quantitative differences in composition of epicuticular waxes between faba bean and French bean could have also affected fungal retention and adhesion on the two host plants. French bean has relatively low epicuticular wax content as compared to faba bean which are waxier, meaning that the bioavailability and efficacy of the fungus was reduced on faba bean. Olleka *et al*. (2009) attributed the lower numbers of spores and subsequent mortality rates of *Bemisia tabaci* (Gennadius) on cabbage treated with *B. bassiana* as compared to those treated on cucumber, egg plant and tomato, to high waxiness of the cabbage leaves. Duetting *et al*. (2003) reported that higher proportions of pea aphids, *Acyrthosiphon pisum* Harris (Homoptera: Aphididae), were killed by *Pandora neoaphidis* (Remaudière and Hennebert) Humber on a line of peas, *Pisum sativum* L. with genetically reduced wax bloom than on a normal wax bloom sister line. They attributed the high infection rates to an increase in adhesion and germination of conidia on the insect cuticle on less waxy plants compared to those with higher levels of wax. Inyang *et al*. (1998a) reported that while epicuticular waxes of oil seed rape appeared not to interfere with acquisition of *M. anisopliae* by larvae of the mustard beetle *Phaedon cochleariae* (Fabricius) (Coleoptera: Cassidae) on the contrary they appeared to enhance

conidial acquisition by larvae fed on the turnip and Chinese cabbage. High degrees of waxiness increase hydrophobic properties of the surface and interfere with conidial adhesion of phyopathogenic fungal spores which are normally favoured by increased hydrophilicity of the substrate (Duetting *et al*., 2003). Waxiness has also been reported to affect spray droplet deposition patterns which in turn affect levels of conidial aggregation on leaf surfaces (Ugine *et al*., 2007).

Many phytochemicals especially allelochemicals and nutrients can modify the physiology and growth of the insect host, thereby affecting its susceptibility to infection. Vega *et al*. (1997) showed that the presence of allelochemicals (catechol and salicylic acid) either on the leaf surface or insect cuticle had an inhibitory effect on the germination rate of the fungal pathogen *I. fumosorosea*. Insect herbivores are also known to sequester antimycotic phytochemicals that help confer resistance to fungal infection (Ramoska and Todd, 1985; Gallardo *et al*., 1990; Poprawski *et al*., 2000). Poprawski *et al*. (2000) found that third-instar nymphs of *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae) sequestered the glycoalkaloid tomatine making them less susceptible to infection.

It can be concluded from this study that temperature and host plant play an important role in the virulence of the entomopathogenic fungi *M. anisopliae* against the leafminer species. Generally *M. anisopliae* isolates ICIPE 18, 84 and 20 performed better in the different temperature ranges tested. For the host plant bioassays, insects reared on faba plants seemed to take longer to succumb to infection. *Liriomyza huidobrensis* reared on faba bean required higher conidial concentrations to succumb to infection compared to those reared on French bean. This finding emphasizes on the need for considering the host plant in developing the optimum formulation for foliar application of mycoinsecticides.

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CHAPTER FIVE

5. Effect of the entomopathogenic fungus *Metarhizium anisopliae* on the leafminer ectoparasitoid *Diglyphus isaea*

5.1 Abstract

The effect of the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) (isolate ICIPE 20) on the leafminer parasitoid *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) was investigated in the laboratory in three sets of experiments. In the first experiment, parasitoids were exposed to four different concentrations of *M. anisopliae* (1 x 10^6, 10^7, 10^8, 10^9 conidia ml^{-1}) on previously treated *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae)-infested faba bean, *Vicia faba* L. (Fabales: Fabaceae), foliage containing 3rd instar larvae. In the second set of experiments, a similar procedure as above was followed except that plants containing 3rd instar larvae were removed after 24 h and held until parasitoid emergence. A third set of experiments were carried out in order to assess the dynamics of parasitoid emergence. In this set-up, sprayed plants containing 3rd instar larvae were removed one, two, three and four days after exposure and held until parasitoid emergence. Results showed that *M. anisopliae* was pathogenic to *D. isaea* causing up to 76% mortality six days after inoculation. Mortality varied with fungal concentration. Infection by *M. anisopliae* also affected *D. isaea* emergence.

5.2 Introduction

The Agromyzid leaf miner genus *Liriomyza* (Diptera) contains some of the most destructive pests of vegetable and floricultural crops world-wide (Spencer, 1973; Parrella, 1982; Minkenberg and Van Lenteren, 1986). *Liriomyza* sp. causes damage by puncturing the leaf surface to feed and to lay eggs into the tissue. When the eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines (Spencer, 1973; Knodel-Montz *et al*., 1985, Ameixa *et al*.,

2007). Biological control is the preferred option for the management of these leafminer pests mainly because chemical control has proven to be ineffective (MacDonald 1991, Weintraub and Horowitz 1995, Murphy and LaSalle 1999, Rauf *et al*., 2000). Biological control using parasitoids and entomopathogenic fungi is among the alternatives being considered. For instance, the larval ectoparasitoid *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) has been used as a biological control agent of leafminers in vegetables and ornamentals in greenhouses (Murphy and LaSalle, 1999; Bazzocchi *et al.*, 2003; Haghani *et al.,* 2007). The potential of entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) for the control of leafminer was recently demonstrated (Migiro *et al*., 2010).

Entomopathogenic fungi are generally considered as safer than chemical insecticides. Their safety to nontarget arthropods was reviewed by Goettel *et al.* (1990), Vinson (1990) and Roy and Pell (2000). Although entomopathogenic fungi have the widest spectra of host ranges [(for instance, *Metarhizium* spp. has been reported on more than 200 hosts (Veen, 1968)], no conclusions should be drawn based on such host lists since many records are based on a single specimen with dubious identification of both host and pathogen, and the fact that host ranges have rarely been verified experimentally (Goettel *et al*. 1990). Furthermore, laboratory studies have shown that different isolates of the same species have varying degrees of specificity (Fargues, 1976; Maniania and Fargues, 1984). Therefore, the effect of an entomopathogenic fungal isolate should be evaluated against non-target organisms before integration as a component of an integrated pest management (IPM) system. The present study therefore investigates the effect of the *M. anisopliae* ICIPE 20 on the parasitoid *D. isaea.*

5.3 Materials and methods

5.3.1 Plants

Faba bean, *Vicia faba* Linnaeus (Fabales: Fabaceae) plants were used for *L. huidobrensis* rearing in the experiments. Plants were grown in a screen house (2.8 m length x 1.8 width × 2.2 m height) in 15 cm pots (three to four plants per pot), using a mixture of manure and soil in a ratio of 1 to 5, respectively, at the International Centre of Insect Physiology and Ecology (*icipe*).

5.3.2 Insects

Liriomyza huidobrensis-infested faba bean plants containing 2-3rd instar larvae were reared at the Animal Rearing and Quarantine Unit at *icipe.* Five hundred two to three day-old mated adult leafminers were released on 60 pots containing two week faba bean plants and allowed to lay eggs for 24 hours. The adults were then aspirated out of the rearing cages and plants containing eggs were held until larvae were at the second-third instar stage. The number of larvae per plant (from six randomly selected plants) ranged from 113 to 145. The parasitoids *D. isaea* were obtained from Dudutech Co., Ltd., Nanyuki, Kenya. They were maintained on *L. huidobrensis* larvae on *V. faba* and later fed on 30% honey solution before delivery to *icipe*. Their sex ratio was 1: 2 for male to female respectively.

5.3.3 Fungal isolates

The fungal isolate used in the study was obtained from the *icipe*'s Arthropod Germplasm Centre. It was cultured on Sabouraud dextrose agar (SDA) in Petri dishes (9 cm) and incubated at 25 ± 2°C in complete darkness. Conidia were harvested from three week old surface cultures. Conidia viability was determined by spread-plating 0.1 ml of 3 x 10^6 conidia ml^{-1} onto 9 cm Petri dishes containing SDA medium. A sterile microscope cover slip (2 x 2 cm) was placed on top of the agar in each plate. Plates were incubated in complete darkness at 25 ± 2°C and examined after 20 h. Percentage germination of conidia was determined from 100 random conidia on the surface area covered by each coverslip under the

light microscope (400X) (Goettel and Inglis, 1997). Each plate represented a replicate with each isolate having four replicates.

5.3.3.1 Effect of *M. anisopliae* on mortality of *D. isaea*

Potted faba bean plants infested with 3rd-instar *L. huidobrensis* larvae were sprayed directly with 10 ml (1 pot was sprayed at a time) of different concentrations (1.0 x 10^6, 1.0 x 10^7, 1.0 x 10^8 and 1.0 x 10^9 conidia ml^{-1}) of *M. anisopliae* isolate ICIPE 20 using Burgerjon's (1956) spray tower. Controls were sprayed with sterile distilled water containing 0.05% Triton X-100. The sprayed plants were then air dried before being transferred into a clean ventilated Perspex cage (300 x 300 x 300 mm). Two pots (each containing three to four infested plants) were used per replicate for each concentration. Fifty *D. isaea* were then introduced into each of the cages containing sprayed potted plants. The experiment was replicated four times and was arranged in a complete randomized design. Potted plants were maintained at 25-27°C, 50-70% RH and 12L: 12D photoperiod. After 24 hours, sprayed plants were replaced with fresh ones and thereafter, foliage was changed on alternate days. Mortality was recorded daily until all parasitoids died. Dead parasitoids were placed on Petri dishes lined with damp sterilized filter paper to allow fungal growth on the surface of the cadaver.

5.3.3.2 Effect of *M. anisopliae* on parasitoid emergence

The same procedure as above was used, except that after 24h, all parasitoids were aspirated from the cages and infested plants were held in the same cages to allow parasitoid or leafminer emergence. The number of emerged parasitoids was recorded. The experiment was replicated four times and repeated at a later stage.

The dynamics of parasitoid emergence was studied in another set of bioassays. A similar procedure as above was followed except that, infected parasitoids were provided with fresh infested potted plants containing 3rd instar *L. huidobrensis*

larvae to parasitize at one, two, three and four days post exposure. Infested plants purposely containing parasitized larvae were held in cages (300 x 300 x 300 mm) to await parasitoid emergence. The experiment was replicated four times for each day and concentration. Emergence of parasitoids was recorded for the different times post-inoculation.

5.3.4 Statistical analysis

Percentage mortality data was corrected for control mortality (Abbot, 1925) and normalized by angular transformation (Sokal & Rohlf, 1981) while data on number of emerged parasitoids was log transformed (x+1) before being subjected to analysis of variance (ANOVA), at 95% level of significance. Student-Newman-Keul's (SNK) procedure was used to separate the means as a post ANOVA procedure (α = 0.05).

5.4 Results

5.4.1 Effect of *M. anisopliae* on mortality of *D. isaea*

Mortality data are presented in figure 5.1. There were no significant differences in mortality among treatments at day one (F = 2.51; df = 4,15; P = 0.0856) and two (F = 2.80; df = 4,15; P = 0.0645) after treatment. However, significant differences were recorded for day 3 (F = 3.15; df = 4,15; P = 0.0456), four (F = 4.66; df = 4,15; P = 0.0121), five (F = 12.55; df = 4,15; P < 0.0001) and six (F = 11.44; df = 4,15; P = 0.0002) after treatment, and this mortality varied with concentration. For instance, the lowest concentration of 1 x 10^6 conidia ml^{-1} caused the lower mortality (38. 56 ± 4.84%) while the higher concentration of 1 x 10^9 conidia ml^{-1} caused the highest mortality (76.28 ± 11.87%) (Fig. 5.1) at six days after treatment. The lowest mortality rate of 12 ± 1.41 % was observed in the control treatment.

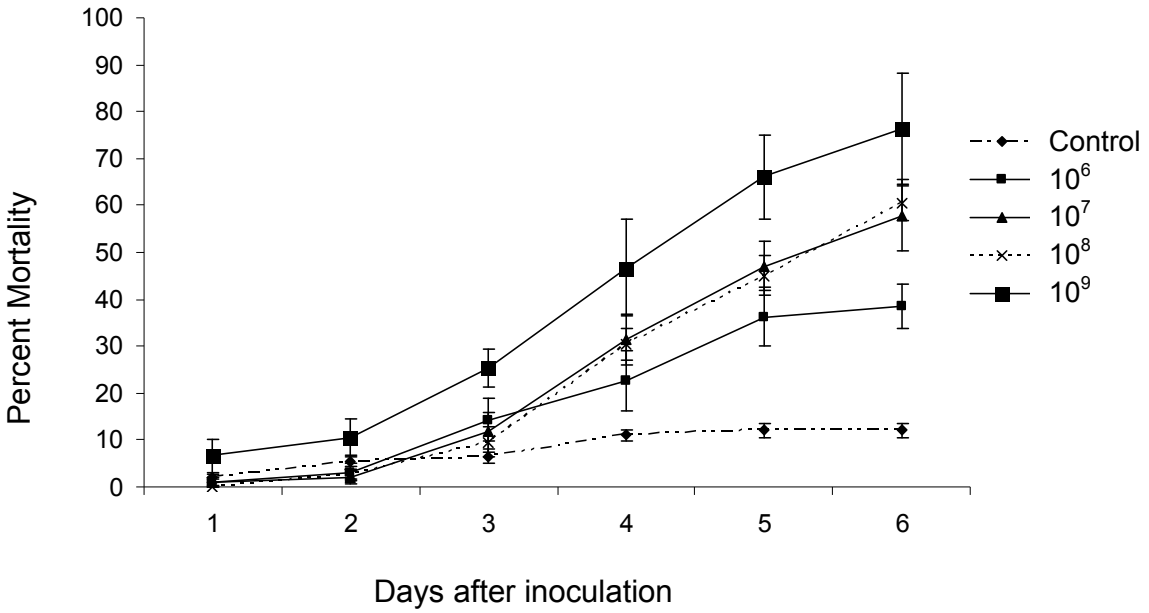

Figure 5.1 Virulence of *Metarhizium anisopliae* isolate ICIPE 20 against the leafminer parasitoid, *Diglyphus isaea* (n=20).

5.4.2 Effect of *M. anisopliae* on parasitoid emergence

The mean number of emerged *D. isaea* individuals was affected by *M. anisopliae* infection and varied with conidial concentration ($F = 2.49$; $df = 4, 315$; $P = 0.0430$) (Table 5.1). The highest number of parasitoid that emerged was recorded in the control treatment (8.80 ± 1.36) while the lowest was recorded at the highest conidial concentration of 1×10^9 conidia ml^{-1} (3.61 ± 0.54) (Table 5.1).

Table 5.1 Efffect of *Metarhizium anisopliae* isolate ICIPE 20 on the number of emerged *Diglyphus isaea* in the laboratory.

Conidial concentration ml^{-1}	Emerged *D. isaea* (mean ± SE)
1×10^6	3.72 ± 0.49ab
1×10^7	5.47 ± 0.79ab
1×10^8	5.30 ± 0.94ab
1×10^9	3.61 ± 0.54b
control	8.80 ± 1.36 a

Means within columns followed by same letter are not significantly different, ANOVA and Student-Newman-Keuls (SNK) comparisons of means, $\alpha = 0.05$. n = 320.

The mean number of emerged parasitoids at different day's post-inoculation is presented in figure 5.2. The number of parasitoids that emerged did not differ significantly among the different concentrations of *M. anisopliae* for day one ($F = 1.12$; df = 3,76; $P = 0.3453$), two ($F = 0.49$; df = 3,76; $P = 0.6917$), three ($F = 1.14$; df = 3,76; $P = 0.3377$) and day four ($F = 0.65$; df = 3,76; $P = 0.5841$), indicating a lack of dose effect on parasitoid emergence. However, the number of emerging parasitoids among the concentrations and control treatments differed significantly for days two ($F = 2.88$; df = 4, 95; $P = 0.0268$), three ($F = 14.15$; df = 4,95; $P < 0.0001$) and day four ($F = 20.77$; df = 4,95; $P < 0.0001$) with no differences being recorded for day one post-inoculation ($F = 1.08$; df = 4,95; $P = 0.3721$) (Fig. 5.2).

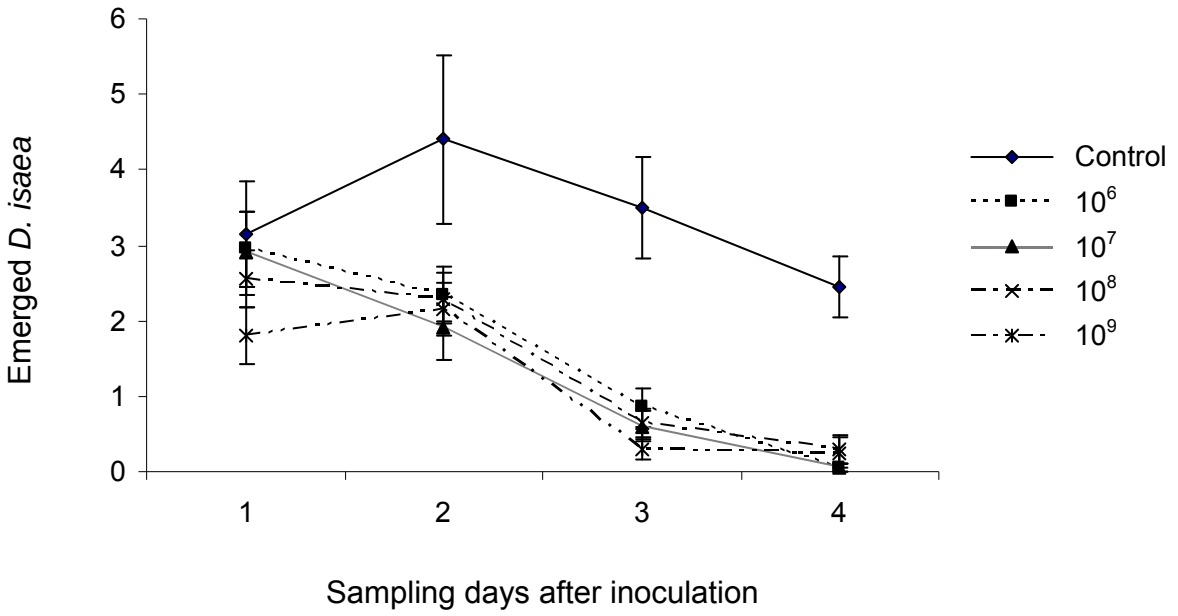

Figure 5.2 Effect of *Metarhizium anisopliae* isolate, ICIPE 20 at four concentrations on emergence of *Diglyphus isaea* at different days post infection (1-4) (n=100).

5.5 Discussion

Results of this study showed that *M. anisopliae* has the potential to infect *D. isaea* and also to affect its emergence. Similar results were reported by James and Lighthart (1994) when evaluating the effect of five entomopathogenic fungi, *M. anisopliae*, *Isaria fumosorosea (=Paecilomyces fumosoroseus)* (Wize) Brown and Smith, *Nomuraea rileyi* (Farlow) Samson and two strains of *Beauveria bassiana* (Balsamo) Vuillemin against the predatory lady beetle, *Hippodamia convergens* Guérin Méneville (Colepotera: Coccinellidae) in the laboratory. With the exception of *Nomuraea rileyi* (Farlow), all the fungal isolates caused mortality from 56 to 95%, and mortality was dose-dependent. Magalhães *et al.* (1988) reported 60% mycosis in adult *Coleomegilla maculata lengi* Timberlake and 35% in adult *Eriopis connexa* Mulsant (Coleoptera: Coccinellidae) following direct application of *B. bassiana.*

Studies have shown that different fungal isolates have varying degrees of specificity. For instance, Poprawski *et al*. (1998) reported that the survival of *Serangium parcesetosum* Sicard (Coleoptera: Coccinellidae), an important predator of whiteflies, was lower when the predators were sprayed with *B. bassiana* than with *I. fumosorosea* and that survivorship was not affected by the dose rate for each pathogen. Thungrabeab and Tongma (2007) reported that *B. bassiana* was non-pathogenic to natural enemies *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)) and *Dicyphus tamaninii* Wagner (Heteroptera: Miridae) as well as the beneficial soil insect, *Heteromurus nitidus* Templeton (Coleoptera: Helopidae) while *M. anisopliae* was pathogenic to *C. carnea* and *D. tamaninii*, with *D. tamaninii* being more susceptible than *C. carnea.*

In conclusion, the *M. anisopliae* ICIPE 20 has adverse effects on the leafminer parasitoid *D. isaea* and can therefore not be used indiscriminately. It might be necessary, however, to determine the timing of the release of the two biological control agents and the concentrations that may be compatible with the parasitoid.

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CHAPTER SIX

6. General discussion, conclusions and recommendations

6.1 Discussion

Liriomyza leafminers especially *L. huidobrensis*, *L. sativae* and *L. trifolii* are economically important pests of a wide range of ornamental and vegetable crops (Spencer, 1973; Waterhouse and Norris, 1987; Murphy and LaSalle, 1999). Their success as cosmopolitan species is attributed to their multivoltinism nature, ability to rapidly develop resistance to chemical pesticides and their polyphagous nature (MacDonald, 1991; Ameixa et al., 2007). Both adults and larvae cause damage to crops. Females puncture leaves to feed and oviposit and when larvae hatch, they tunnel within the leaf tissue forming damaging and disfiguring mines. Extensive puncturing and larval mining can lead to total crop losses (Spencer, 1973; 1990).

Both smallholder and large-scale producers worldwide have largely relied on chemical insecticides for leafminer management. However, indiscriminate and excessive use of chemicals has resulted in insecticide resistance of flies as well as elimination of their natural enemies (MacDonald, 1991; Weintraub and Horowitz, 1995; Murphy and LaSalle, 1999). This has led to an upsurge in research aimed at more biorational management alternatives. There has been increased interest in the use of microbial control agents against agricultural (Dimbi *et al*., 2003; Ekesi *et al*., 2007; Bugeme *et al*., 2009) veterinary (Kaaya and Munyinyi, 1995; Kaaya *et al.*, 1996; Maniania, 1998) and medically important pests (Fetter-Lasko and Washino, 1983; Watson *et al*., 1995, Renn *et al*., 1999). However, there are only two reports in literature documenting the use entomopathogenic fungi against leafminers (Borisov and Ushchekov, 1997; Bordat *et al*., 1988) but these studies were limited to the screening of fungal isolates in the laboratory against puparia and not adults. The objective of this

study was therefore to investigate the potential of entomopathogenic fungi *B. bassiana* and *M. anisopliae* for the control of the invasive *L. sativae*, *L. trifolii* and *L. huidobrensis*.

In the first experiment (Chapter 2) the pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* isolates against adult *Liriomyza huidobrensis* was investigated in the labaratory. One of the virulent isolates was further used in the evaluation of the autoinoculation device in field cage experiments. Results confirm the pathogenicity of the entomopathogenic fungi *M. anisopliae* and *B. bassiana* to *L. huidobrensis* with the discovery of highly virulent isolates that could be considered for further development as microbial control agents for leafminer management. An autoinoculation device was constructed from locally available material making it cost effective and affordable to small scale farmers. It was shown that leafminer flies were attracted to the device and that they were able to pick up a lethal dose of inoculum, resulting in higher adult mortality. This is an important achievement since use of autoinoculation devices generally require small amounts of spores compared to inundative applications which may require up to 2kg of conidia per ha. Also, conidia have been found to survive for longer periods in the devices in the field (Maniania, 1998; Dimbi *et al*., 2003) making this method economical since there is no need for frequent applications. One of the constraints in the use of biological control agents under field conditions is their persistence. Therefore the use of devices that can prolong their efficacy is important since the frequency of pathogen application will be reduced which in turn will translate to cost reduction.

Studies on the effect of fungal infection on feeding and oviposition (Chapter 3) showed that *M. anisopliae* can significantly reduce feeding and oviposition by *L. huidobrensis*. However, reductions in punctures and eggs generally occurred only after 72 h post-inoculation. The lag of time between inoculation and the onset of the fungal infection of flies, as expressed by the decrease in feeding and oviposition, was due to the fact that entomopathogenic fungi are slow-killing

agents. The latter result implies that there is need for an integrated approach and timely application of the fungus before onset of peak feeding and oviposition in leafminers. Host plant did not have any effect on the feeding but had an influence on the oviposition, with faba bean harboring greater number of eggs in both the control and the *M. anisopliae* treatments. Furthermore, insects reared on faba bean were less susceptible to fungal infection than those reared on French bean and snow pea. The host plant of a phytophagous insect can influence the host insect-pathogen relationships such as influencing pathogen acquisition and infection rates (Ekesi *et al*., 2000; Cory and Hoover, 2006).

Temperature and host plant play an important role in the virulence of the entomopathogenic fungus *M. anisopliae* against the leafminer species (Chapter 4). *Metarhizium anisopliae* isolates ICIPE 18, 84 and 20 performed better under the different temperature regimes tested, hence, making them ideal for control of the three leafminer species in a wider range of environments. In bioassays with different host plants it was observed that insects reared on faba bean plants seemed to take longer to succumb to infection. Higher conidial concentrations were required in order to kill *L. huidobrensis* reared on faba bean compared to those reared on French bean. For both *L. sativae* and *L. trifolii* however, no differences in conidial concentrations were recorded between the two host plants. This finding emphasizes on the need for consideration of the host plant in developing the optimum formulation for foliar application of mycoinsecticides.

Metarhizium anisopliae ICIPE 20 was found to be pathogenic to the leafminer parasitoid *D. isaea* (Chapter 5). Consequently, the two biological control agents can not be used altogether. Therefore, it might be necessary to determine the timing of the release of the two biological control agents as well as concentrations that may be compatible with the parasitoid.

6.2 Conclusions

1. The entomopathogenic fungi *M. anisopliae* and *B. bassiana* are pathogenic to leafminers in the laboratory. The autoinoculative device developed in this study resulted in effective inoculation of flies which subsequently resulted in effective control.
2. Infection by *M. anisopliae* significantly reduces feeding and oviposition by *L. huidobrensis* and reductions in punctures and eggs generally occured after 72 h post-inoculation.
3. Temperature and host plant play an important role in the virulence of *M. anisopliae* against leafminer species. Most isolates were more virulent at 25 and 28° C and disease development increased with increased temperature. Virulence of *M. anisopliae* was affected by host plant species. Insects reared on faba bean plants seemed to take longer to succumb to infection. Higher conidial concentrations were required in order to kill *L. huidobrensis* reared on faba bean compared to those reared on French bean. For both *L. sativae* and *L. trifolii* however, host plant did not affect conidial concentrations.
4. *Metarhizium anisopliae* was pathogenic to *D. isaea* causing up to 76% mortality at six days after inoculation and also affected *D. isaea* emergence suggesting that the two biological control agents cannot be used altogether.

6.3 Recommendations

1. The evaluation of the autoinoculation device was based on the delivering of conidia into fly populations in field cages. All flies died from fungal infection, which is an indication of successful contamination of flies through the device. However, the study did not address the fly-to-fly

transmission of the infection, which is fundamental to the success of this technique. Further studies are required to determine whether fungus-infected flies could transfer infection to healthy flies. There is also need to establish the number of devices required per hectare for successful management of leafminers. Fungal persistence in the devices over time in the field needs to be established.

2. The study of tritrophic interactions between leafminers, host plant and the entomopathogenic fungi will be important in the understanding of the differences in efficacy of *M. anisopliae* on insects feeding on different host plants. This will guide the development of an entomopathogen formulation that will be adequate depending on the targeted host plant.
3. The search for more host-specific isolates is necessary in order to minimize adverse effects of entomopathogenic fungi on non target insects. It will also be necessary to determine the timing of the release of entomopathogenic fungi and the parasitoids for maximum additive effectiveness of the two biological control agents. The compatibility of entomopathogenic fungi with other leafminer parasitoids should also be evaluated in future.

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