PROSPECTS OF FUNGAL ENDOPHYTES IN THE CONTROL OF *LIRIOMYZA* LEAFMINER FLIES (DIPTERA: AGROMYZIDAE) IN COMMON BEAN *PHASEOLUS VULGARIS* LINNAEUS (FABALES: FABACEAE) UNDER FIELD CONDITIONS

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2018

Prospects of fungal endophytes in the control of *Liriomyza* leafminer flies (Diptera: Agromyzidae) in common bean *Phaseolus vulgaris* Linnaeus (Fabales: Fabaceae) under field conditions

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A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Zoology (Agricultural Entomology) in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University

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DEDICATION

This thesis is dedicated with deepest appreciation to my daughter Kelly Wanjiru who constantly reminded me that I have to complete my homework, my parents Mr. John Gathage and Mrs. Grace Gathage and my siblings for their love, generosity and support that was always my inspiration and strength during the entire period of hard work and various challenges.

AKNOWLEDGEMENTS

I am very grateful to God for immense blessings and care in the course of my studies. I am also grateful to the International Centre of insect Physiology and Ecology (ICIPE) and Jomo Kenyatta University of Agriculture and Technology (JKUAT) for providing the necessary support and the opportunity to study in the two institutions. This study was conducted with the financial support from the Federal Ministry for Economic Cooperation and Development, Germany (BMZ), through the German Federal Enterprise for International Cooperation (GIZ) (Grant number: 09.7860.1-001.00; Contract number: ICIPE 81121261). I am grateful to the Leafminer Project for providing research facilities under Dissertation Research Internship Programme (DRIP) of International Center of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya. I highly acknowledge this financial support which helped me to make another fruitful step in life.

I sincerely thank my supervisor from JKUAT, Dr. Zipporah Lagat for the moral support and ensured that no delays occurred to hinder my progress. This work would not have been possible without the immense support and superb supervision from my ICIPE supervisors Dr. Komi Fiaboe and Dr. Nguya Maniania. I highly appreciate your kind mentorship, kind advice, patience and keenness to details and the constant little push which I greatly needed. I am extremely blessed to have had an opportunity to learn from you and gain the research, leadership and management skills.

My heartfelt appreciation also goes to my advisor Dr. Komivi S. Akutse of ICIPE for his immense guidance and support in the data analysis and interpretation. I am also very grateful to Dr. Daisy Salifu for her assistance in the statistical analysis. She constantly guided and encouraged me and gave me hope when the going got tough. I am grateful for the unwavering support of the staff of the Leafminer Project Phase II and Arthropod Pathology Unit; it was a pleasant and fruitful experience working with you all. Specifically, I am highly indebted to Mr. Sospeter Wafula and Ms. Gloria Muthoni for their technical support in laboratory techniques. I would like to profoundly thank Prof. Zebeitz for offering me the opportunity to attend the summer school to train on biological control at the University of Huohenhem in Germany. It was really a great experience and exposure and the training left a

mark in my field of research. The research and data analysis skills learnt created a firm foundation and inspired me to move on in the field of research. Prof. Zebeitz's wise counsel and encouragement gave me hope of attaining the highest academic level.

The field work would not have been complete without the support of the driver Mr. Mucheru Gachugu and the enumerators Mr. P. Githui, Mr. A. Macharia and Mr. R. Muchoki for their help and assistance. I also cannot forget to appreciate my only classmate Miss Sylvia Wanambisi for her great moral support and encouragement when challenges came knocking and Faith Nyamu of Leafminer project Phase II for her moral support and technical advice.

I proudly thank all my family members for their inspiration and support for the whole of my academic life. Special thanks go to my daughter Kelly for her understanding and providing an enabling environment for me to realize my academic dream. She constantly reminded me that I have to complete my 'homework' so that I will be able to graduate soon.

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LIST OF ABBREVIATIONS AND ACRONYMS

AD	Autodissemination device
ANOVA	Analysis of variance
BCAs	Biological control agents
DRIP	Dissertation of Research Internship Program
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
EUROPHYT	European Union Notification System for Plant
FAO	Food and Agriculture Organization
FVO	Food and Veterinary Office
GLM	Generalized linear model
ICIPE	International Centre of Insect Physiology and Ecology
IPM	Integrated pest management
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEPHIS	Kenya Plant Health Inspectorate Service
KNBS	Kenya National Bureau of Statistics
LMF	Leafminer flies
Masl	Meters above sea level

PDA	Potato dextrose agar
RASFF	Rapid Alert System for Food and Feed
RCBD	Randomized in complete block design
RH	Relative humidity
SDA	Sabouraud dextrose agar
SNK	Student Newman Keuls
UNCED	United Nation Conference on Environment and Development
USA	United States of America

ABSTRACT

Liriomyza leafminers species are exotic pests of horticultural crops in Africa. The most economically important species include *Liriomyza sativae* (Blanchard), *L. trifolii* (Burgess) and *L. huidobrensis* (Blanchard) (Diptera: Agromyzidae) which are major pests of many vegetables and ornamental crops worldwide. In Kenya, production of horticultural crops is severely constrained by infestation of *Liriomyza* leafminer flies (LMF). As a result, farmers increasingly use synthetic chemical insecticides and spray more frequently in response to damage by key pests such as LMF which has led to development of pests' resistance to synthetic pesticides, elimination of natural enemies, environmental contamination, and health risks due to pesticide residues and increased costs of production costs. These challenges have stimulated an increased interest in the use of biological control strategies. Parasitoids and fungal entomopathogens are being considered as part of an integrated strategy for leafminer management in Kenya. The objective of this study was to evaluate the prospects of endophyte isolates of *Beauveria bassiana* (Balsamo) Vuillemin G1LU3 and *Hypocrea lixii* Patouillard F3ST1 for the control of *Liriomyza* leafminer in common bean *Phaseolus vulgaris* (Linnaeus) (Fabales: Fabaceae) crops through seeds inoculation under field conditions. Autodissemination device (AD) treated with conidia of *Metarhizium anisopliae* (Metschnikoff) Sorokin ICIPE 20 was also added as a treatment. Leafminer flies infestation was not significantly different during the long rains season (season 1) but was higher in the controls than in endophtyte treatments at both sites during the short rains season (season 2). Three key *Liriomyza* species [(*L. huidobrensis* Blanchard, *L. sativae* Blanchard and *L. trifolii* (Burgess)] and six parasitoid species [(*Opius dissitus* Muesebeck, *Phaedrotoma scabriventris* Nixon, *Diglyphus isaea* Walker, *Neochrysocharis formosa* Westwood, *Hemiptarsenus varicornis* Girault and *Halticoptera arduine* (Walker)] were identified during the trials. The effects of endophytes on leafminer infestation, number of pupae, leafminer flies and parasitoids emergence and yield were the parameters evaluated. The experiments were conducted in the laboratory and field. Field trials were carried out for two seasons (long and short rains seasons) in two sites (Sagana and Naromoru, Central province of Kenya). Both isolates successfully colonized different parts of *P. vulgaris* plants, although the colonization was higher with *H. lixii* F3ST1 than *B. bassiana* G1LU3 at both sites. The mean number of

pupae from the infested leaves varied between 141-252 and 331-416 in endophyte and control treatments, respectively, during the long rains season (season 1) and from 110-223 and 366-523, respectively, in endophyte and control treatments during the short rains season (season 2). There were no significant differences among the treatments in the number of parasitoids that emerged from pupae. Higher yield of *P. vulgaris* seeds was obtained in endophyte treatments than in control treatments. The inclusion of AD treatment did not have significant effect on all the parameters evaluated, except yield. Results of the present study suggest that both fungal isolates hold potential for pest management and could be considered for the control of leafminer flies. Improvement of the autodissemination device traps is necessary by using materials which could withstand harsh environment conditions in the field. Further work should be done to establish colonization of *P. vulgaris* plants in the subsequent generations.

CHAPTER ONE
INTRODUCTION

1.1 Background of the study

The *Liriomyza* leafminer flies (LMF) are invasive species to Africa and believed to be of neotropic origin. The family Agromyzidae (Diptera) contains some of the world's most destructive pests of vegetable and floricultural crops (Parrella, 1982; Minkenberg and Van Lenteren, 1986). *Liriomyza* species (Diptera: Agromyzidae) are exclusively plant feeders and are virtually ubiquitous (Spencer, 1989). *Liriomyza sativae* (Blanchard), *Liriomyza trifolii* (Burgess) and *Liriomyza huidobrensis* (Blanchard) are the major pest species that pose a huge threat to horticultural field crops worldwide (Reitz & 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 (Liu *et al*., 2008). They attack a wide variety of agricultural crops, particularly vegetables and ornamental plants worldwide (Murphy & Lasalle, 1999). The three *Liriomyza* species of leafminers that pose threat to horticultural industry in many African countries include *L. sativae, L. trifolii* and *L. huidobrensis.*

In Kenya, leafminer pests have recently become a major problem in the horticultural industry. The damage by LMF has been recorded on various crops. *L. huidobrensis* has been reported to cause damage on vegetables and other ornamental plants such as passion fruit (*Passiflora edulis),* Sims (Malpighiales: Passifloraceae), beans (*Phaseolus vulgaris* Linnaeus (Fabales: Fabaceae)), snow peas (*Pisum sativum* Linnaeus Var. Saccharatum (Fabales: Fabaceae)) and gypsophila (Gypsophila spp. (Caryophyllales: Caryophyllaceae)) (KEPHIS, 2005). *L. sativae* has been recorded on tomatoes, passion

fruits, cucumber (*Cucumis sativus* Linnaeus (Cucurbitales: Cucurbitaceae)) and cowpea (*Vigna unguicalata* (Linnaeus Walp (Fabales: Fabaceae)).

Liriomyza trifolii has been recorded in beans (*Phaseolus vulgaris* Linnaeus (Fabales: Fabaceae)) in Thika (Kabira, 1985), on tomatoes in Voi area west of Mombasa (Spencer, 1985), okra (*Abelmoschus esculentus* (Linnaeus Moench (Malvales: Malvaceae)), Sunflower (*Helianthus annuus* Linnaeus (Asterales: Compositae)) at Hola irrigation scheme, onions (*Allium cepa* Linnaeus (Asparagales: Alliaceae)) and Courgettes (*Cucurbita pepo* Linnaeus (Cucurbitales: Cucurbitaceae)).

Large and small scale farmers persistently use synthetic chemical pesticides such as carbamates, organophosphates and pyrethroids (Murphy & La Salle, 1999) to manage leafminer flies. However none of the insecticides have managed to completely control LMF at recommended doses. The natural and biological control of pests and diseases affecting cultivated plants has gained much attention in the past decades as a way of reducing the use of chemical products in agriculture. The United Nations Conference on Environment and Development (UNCED), held in Rio de Janeiro in 1992, recognized integrated pest management, based on biological control, as a key element in reversing agriculture's hazardous dependence on synthetic chemical pesticides and establishing a more environmentally friendly paradigm. Among the options is one that revolves around the use of arthropod pathogens. Microorganisms (bacteria, fungi, viruses, nematodes and protozoa) cause diseases in arthropod populations in nature and thereby contribute to natural regulation of pest populations. Consequently, they can be developed as biopesticides.

The role of endophytic fungi in the control of insects has been the subject of several reviews (Saikkonen *et al*., 1998; Carroll, 1995; Breen, 1994). Integrated Pest Management (IPM) strategies have greatly been embraced worldwide as an effective mean of pest management. Biological control through the use of parasitoids and fungal

entomopathogens is being considered as part of an integrated strategy for LMF management in Kenya (Migiro *et al*., 2010).

1.2 Statement of the problem

Leafminer flies punctures and mines reduce the quality of high value horticultural crops in addition to reducing the photosynthetic ability of the plant (Kox *et al*., 2005) and create entry point for pathogenic micro-organisms which may lead to total crop losses. When leafminers damage crops by puncturing the leaf surface to feed on exuding sap, they also lay eggs into the leaf tissue (Knodel-Montz *et al*., 1985). When eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines. Due to difficulty in the control of leafminer flies, they are listed as regulated pests in the EU Plant Health Directive 2000/29. Large and small scale farmers persistently use synthetic chemical pesticides to manage LMF. Not only has the indiscriminate use of pesticides affected the management of *Liriomyza* leafminer flies and adversely killed their associated natural enemies, but it has also presented a challenge to farmers in developing countries exporting to the European Union. Market access of vegetables and flowers from Kenya, for example, is limited by the presence of pesticide residues (Maximum Residue Limit) above the set limits (RASFF, 2014). The effect of indiscriminate use of broad-spectrum pesticides has dogged their effectiveness because they kill both insect pests and beneficial insect like parasitoids. Therefore, there is great need for an environmentally sustainable alternative to synthetic chemical pesticides.

1.3 Justification of the study

Both large and small scale farmers mainly use synthetic chemical insecticides to control LMF. In many cases pesticides are not effective due to the development of resistance, elimination of natural enemies and pose health risks due to synthetic chemical pesticide residues. Developed resistance to synthetic chemical pesticides make it a bother to farmers since the most destructive stage of the pest (larvae) cannot be killed by these synthetic chemical insecticides as well as the high mobility of the adult flies which make it possible to escape the application.

Biological control through the use of parasitoids and fungal entomopathogens is being considered as part of an integrated strategy for LMF management in Kenya. A two-pronged approach is being considered: the first targets the adult stage by using an auto dissemination device treated with fungal conidia and the second approach consists of interfering with life history of the LMF through endophytic colonization of the host plant. Fungal resistance to herbivores represents an environmentally sustainable alternative to pesticides that has experienced reasonable success in agricultural applications. The aim of the present study was therefore to evaluate the prospects of these two fungal endophytes (*Beauveria bassiana* GILU3 and *Hypocrea lixii* F3ST1) for the control of LMF on *Phaseolus vulgaris* L. (Fabales: Fabaceae) crop under field conditions, in terms of LMF infestation levels and yield, through seed inoculation technique. The study also investigates the effects of fungal treatments on LMF associated parasitoids.

1.4 Hypotheses

1. There is no effect of fungal endophytes, *B. bassiana*, G1LU3 and *H.lixii*, F3ST1 in the management of *Liriomyza* LMF in *P. vulgaris* under field conditions.
2. There is no effect of autodissemination of *M. anisopliae,* ICIPE 20 in management of *Liriomyza* LMF in *P. vulgaris* under field conditions.
3. There is no compatibility between fungal endophytes, *B. bassiana*, G1LU3 and *H.lixii*, F3ST1 and autodissemination of conidia of *M. anisopliae,* ICIPE 20 with parasitoids strategy in management of *Liriomyza* LMF in *P. vulgaris* under field conditions.

1.5 Objectives of the study

1.5.1 General objective

To evaluate the prospects of fungal endophytes in the management of *Liriomyza* LMF in common bean *P. vulgaris* under field conditions.

1.5.2 Specific objectives

1. To determine the effectiveness of fungal endophytes, *B. bassiana*, G1LU3 and *H. lixii*, F3ST1 in the management of *Liriomyza* LMF in *P. vulgaris* under field conditions.
2. To determine the effectiveness of autodissemination of *M. anisopliae*, ICIPE 20 in management of *Liriomyza* LMF in *P. vulgaris* under field conditions.
3. To determine the compatibility between fungal endophytes, *B. bassiana*, G1LU3 and *H. lixii*, F3ST1 and autodissemination of conidia *M. anisopliae*, ICIPE 20 with parasitoids strategy in management of *Liriomyza* LMF in *P. vulgaris* under field conditions.

1.6 The limitations of the study

High infestation of bean plants by bean flies was pronounced in the second to third week after sowing the seeds and flowering time in the long and short rains seasons. It led to withering and even drying of few bean plants. High infestation of bean plants by black bean aphids (*Aphis fabae*) which led to yellowing and curling of leaves. During the dry months, the black bean aphids (*Aphis fabae*) infestation was high leading to poor development of the plants and pods which affected the yield. This was more serious in Sagana location than in Naromoru.

High infestation of bean plants by white flies was another great challenge. This was a critical problem especially at Sagana location in the flowering stages which led to blackening of the leaves due to their waste in the long and short rains seasons. This affected the rate of photosynthesis leading to poor bean plants development. An

alternative control strategy involving an application of neem-based pesticide (Neemroc) was used which led to a reduction in other insect pests without harmful effects on the endoparasitoids. Disease like halo bright infected the bean plants. The farmer's cows on few occasions ate a few bean plants due to the poor handling of the cows which are grazed outside the cow sheds by the farmers in both locations.

Environmental factors like wind, rainfall and sun heat effect on autodissemination devices was noted which affected the performance of the autodissemination device. In Naromoru there was strong wind due to its high altitude which would lead to the bean plants being swayed to the side hence lying on the ground. The strong heat and rain led to breakage of the plastic containers of the autodissemination devices. This led to exposure of the velvet cloth holding the *M. anisopliae* conidia to the wind.

Unreliability of rainfall affected the rate of infestation by other pests like aphids. In order to maintain good development of bean plants, irrigation had to be done leading higher cost of production. Heavy rainfall with hail stones was a big challenge especially in the short rains season. This led to tearing of leaves, breaking of pods leading to release of the bean seeds on the ground and breakage and falling of the plastic covers of the autodissemination devices.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction

Liriomyza leafminer flies (LMF) attack a wide variety of agricultural crops, particularly vegetables and ornamental plants worldwide (Murphy & La Salle, 1999). In Kenya, LMF have recently become a major problem in the horticultural industry, the largest sector in Kenyan economy generating annual revenue of US$ 2 billion in 2013 (KNBS, 2014). Yield losses of 20-100% have been reported, depending on crop and location (EPPO, 2006; Chabi-Olaye, 2008). In addition, LMF are listed as quarantine pests in overseas markets, especially the European Union (EPPO, 2006), and therefore prevent access of Kenyan horticultural products to new market opportunities (KEPHIS, 2006). In 2013, interceptions due to *Liriomyza* species comprised 37% of interceptions due to presence of harmful organisms (FVO, 2013; EUROPHYT, 2014). This demonstrates the high levels of *Liriomyza* leafminer losses and their economic importance in the horticultural sector. Large and small scale farmers persistently use synthetic chemical pesticides to manage LMF.

However, none of the synthetic chemical insecticides have managed to completely control LMF because of the resistance LMF have developed to major groups of insecticides, as reported in some vegetable productions parts of Central Kenya (Price & Nagle, 2002; Gitonga *et al*., 2010). Moreover, indiscriminate use of pesticides affects adversely LMF associated natural enemies and the concerns over pesticide residues. IPM strategies have greatly been embraced worldwide as an effective mean of pest management. Biological control has been recognized as a key element in reversing agriculture's hazardous dependence on synthetic pesticides and establishing a more environmentally friendly paradigm (UNCED, 1992).

The use of fungal pathogens is among the strategies being explored for pest management. A two-pronged approach is being considered for their application in the

field: the first consists of autodissemination of conidia targeting the adult stage (Migiro *et al*., 2010) and the second one uses fungal pathogens as endophytes (Clay 1990; Vega *et al*., 2009). Fungal endophytes are microorganisms that colonize internal plant tissues without having any symptomatic effects on the host plant (Rodriguez *et al*., 2009). This was also reported by (Azevedo, 1998) that, endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches and stems, showing no apparently harm to the host. Clay (1990) reported that insect biocontrol may be improved by the development of fungal endophytes artificial inoculation techniques.

Recently, Akutse *et al*., (2013) demonstrated that some fungal isolates were able to endophytically colonize bean plants and confer them greater resilience to *L. huidobrensis* infestation. *Hypocrea lixii* Patouillard (Hypocreales: Hypocreaceae) isolate F3ST1 and *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) isolate GILU3 outperformed the other fungal isolates and were proposed for further studies.

2.2 Host plant (*Phaseolus vulgaris*)

Phaseolus vulgaris, the common bean (also known as the string bean, field bean, flageolet bean, French bean, garden bean, green bean, haricot bean, pop bean, or snap bean), is a herbaceous annual plant grown worldwide for its edible dry seed (known as just "beans") or unripe fruit (green beans). Its leaf is also occasionally used as a vegetable and the straw as fodder. Its botanical classification, along with other *Phaseolus* species, is as a member of the family *Fabaceae*, most of whose members acquire the nitrogen they require through an association with rhizobia, a species of nitrogen-fixing bacteria. The common bean is a highly variable species that has a long history of cultivation. Beans are grown in every continent except Antarctica. Brazil and India are the largest producers of dry beans, while China produces, by far, the largest quantity of green beans. Worldwide, 23 million tonnes of dry common beans and 17.1 million tonnes of green beans were grown in 2010 (FAO, 2010).

The common bean (*Phaseolus vulgaris)* is the most important food legume which is grown worldwide. It is high in starch, protein, dietary fiber (both soluble and insoluble fiber), and is an excellent source of iron, potassium, selenium, molybdenum, thiamine, vitamin B6 and folic acid. Common bean therefore plays a key role in the nutrition and health of the producers and consumers providing employment opportunities and serving as a major source of income generation through local market and export. In Kenya it is cultivated on an estimated 700,000 ha (FAO, 2010). An average yield of 750 kg/ha is realized, against a potential of 1500 kg/ha.

Dry beans will indefinitely stay for a long time if stored in a cool, dry place, but as time passes, their nutritive value and flavor degrade and cooking times lengthen. Dried beans are almost always cooked by boiling, often after being soaked in water for several hours. While the soaking is not strictly necessary, it shortens cooking time and results in more evenly textured beans. In addition, soaking beans removes 5 to 10% of the gas-producing sugars that can cause flatulence for some people. The methods include simple overnight soaking and the power soak method in which beans are boiled for three minutes and then set aside for 2 - 4 hours. Before cooking, the soaking water is drained off and discarded. Dry common beans take longer to cook than most pulses: cooking times vary from one to four hours, but are substantially reduced with pressure cooking. Salt, sugar, and acidic foods such as tomatoes may harden uncooked beans, resulting in seasoned beans at the expense of slightly longer cooking times. Dry beans may be bought cooked and canned as refried beans, or whole with water, salt, and sometimes sugar.

Liriomyza leafminers attack a wide range of vegetable and horticultural crops (Waterhouse & Norris, 1987; Murphy & La Salle, 1999; Belokobylskij *et al*., 2004) and have also been recorded on several wild hosts (Spencer, 1973, 1985). Leafminers are able to colonize a wide range of plants (primarily although not exclusively Solanaceae, Leguminosae, Cucurbitaceae and Asteraceae). The most common species, *L. sativae*, *L. trifolii* and *L. huidobrensis* feed on a wide range of plants. Main host plants include

cabbage and other brassicas (cruciferous crops), okra, onion, pigeon pea, bell pepper, cucumber, pumpkin, cowpea, potato, passion fruit, tomato and common bean.

The major constraints to bean production include diseases, soil fertility, insect pests and low erratic rainfall (Otsyula *et al*., 1998). Among the insect pests, African bollworm, Helicoverpa spp., thrips, white flies, leafminer flies and aphids are considered the most important pests contributing to low production and poor quality yield.

2.3 *Liriomyza* leafminer flies

Liriomyza leafminer species are exotic pests of horticultural crops in Africa and have invaded large parts of the continent from the New World (Murphy & La Salle, 1999). The most economically important species include *L. sativae*, *L. trifolii* and *L. huidobrensis* (Chabi-Olaye *et al.*, 2008). *Liriomyza* species are listed as quarantine pests in overseas markets, especially the European Union (EPPO, 2006), and therefore prevent access of Kenyan horticultural products to new market opportunities (KEPHIS, 2006). Economic importance is also due to difficulty in their control. Adults may rapidly develop pesticide resistance (MacDonald, 1991) and *Liriomyza* larvae are inaccessible to many pesticides because they develop inside leaves and pupate in the soil. LMF are able to colonise a wide range of plants (primarily although not exclusively Solanaceae) hence they are abundant in the field. They are listed as regulated pests in the EU Plant Health Directive 2000/29 (EU, 2000).

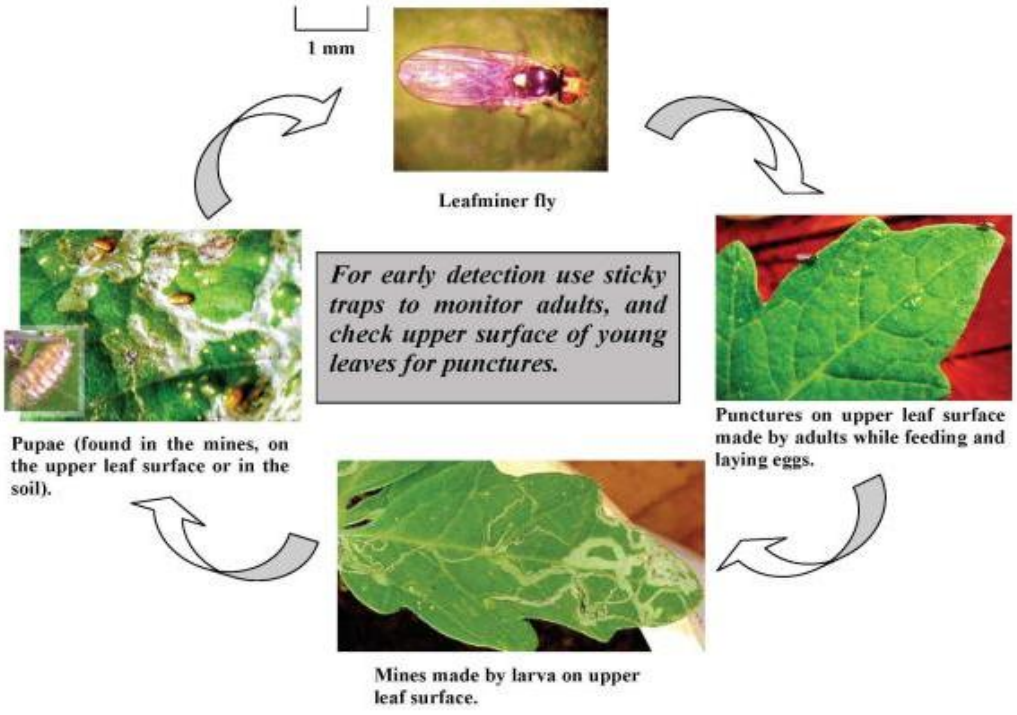

Figure 2.1: Life cycle of *Liriomyza huidobrensis* © A. M. Varela, ICIPE

Female leafminers flies damage crops by puncturing the leaves surface of the host plants with their ovipositor, causing wounds which serve as sites to lay eggs into the leaf tissue and feed on exuding sap (Knodel-Montz *et al.*, 1985). The life cycle has 4 stages, eggs, larvae, pupae and adults (Figure 2.1). 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 (Parrella, 1982).

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). When eggs hatch, the larvae tunnel within the leaf tissue forming damaging and disfiguring mines (Knodel-Montz *et al.*, 1985; Ameixa *et al.*, 2007). Leaf punctures and mines reduce the quality of high value

horticultural crops in addition to reducing the photosynthetic ability of the plant (Foster & Sanchez, 1988).

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 & La Salle, 1999). Heavy attack leads to large-scale necrosis of leaf tissue, eventual shriveling of the whole leaf and may result in complete defoliation of crops. During outbreaks, severe infestations from both adult puncturing and larval-mining can lead to total crop losses (Spencer, 1990).

Figure 2.2: *Liriomyza* leafminer flies pupae on leaves © A. M. Varela, ICIPE

The pupae are distinctly segmented, oval shaped narrowing at the ends (Figure 2.3). 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 (Parrella, 1987).

Figure 2.3: Pupa of the American serpentine leafminer, *Liriomyza trifolii* **(Burgess). Photograph by Lyle J. Buss, University of Florida**

Adults 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. *L. trifolii* has a more grayish upper thorax with much of the head capsule behind the eye being mostly yellow. *L. 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.

Figure 2.4: Adult American serpentine leafminer, *Liriomyza trifolii* **(Burgess). Photograph by Lyle J. Buss, University of Florida.**

Figure 2.*5*: Adult vegetable leafminer, *Liriomyza sativae* (Blanchard). Photograph by Lyle J. Buss, University of Florida.

Figure 2.6: Adult Pea leaf miner, *Liriomyza huidobrensis* (Blanchard). Photograph by Lyle J. Buss, University of Florida.

2.4 Geographical Distribution of LMF

Figure 2.7: Geographical distribution of *Liriomyza* leafminer flies in Africa (red marked) © www.infonet – biovision.org

Leafmining flies are serious pests of vegetables and ornamental plants worldwide. Major species of LMF in Africa are: the serpentine leafminer (*Liriomyza trifolii*), vegetable leafminer (*L. sativae*), cabbage leafminer (*L. brasslike*) and pea leafminer (*L. huidobrensis*).

In Africa it is reported from Egypt, Ethiopia, Kenya, Mozambique, Zimbabwe, Cape Verde and Senegal (Chabi-Olaye *et al*., 2008). *L. sativae* is a typical American pest, whereas the typical leafmining fly in Europe is *L. bryoniae*. *L. trifolii* is common on tomato in America and Europe, and *L. huidobrensis* is occasionally reported on tomato in America but damage has been recorded mainly on ornamentals (Varela *et al*., 2003). *L. trifolii* and *L. sativae* are closely related with similar appearance and overlapping host ranges. In Africa, *L. trifolii* has been reported in several countries, including Kenya, Mauritius, Reunion, Senegal, South Africa and Tanzania (Chabi-Olaye *et al*., 2008).

In Kenya, *L. trifolii* was introduced to Kenya in the late 1970s through chrysanthemum cuttings from Florida (USA) (Spencer, 1985). Since then, *Liriomyza* species have been found throughout the country, attacking vegetables and ornamental plants. *L. huidobrensis* is currently a serious pest of ornamentals and passion fruits. *L. sativae* was recently recorded in Kenya. *L. brassicae* has also been reported for many years as a pest on brassicas and legumes, but in general, the damage done to mature crops is largely superficial.

2.5 Fungal entomopathogens

Entomopathogens cause disease in insects through the effects of infection, parasitism and/or toxaemia (Lacey & Brooks, 1997). Some of the entomopathogens that have been reported to infect leafminers include nematodes, bacteria and fungi. The potential of entomopathogenic fungi as effective biological control agents for dipterans leafminers has been demonstrated. One of the bio-control strategies based on fungal entomopathogens consists on disseminating the pathogen among target pest populations by using devices that attract insects to baited stations where they are contaminated with the pathogen and then return to the environment where they can transmit the pathogen to healthy individuals (Vega *et al*., 2007). Such an approach was evaluated against *L. huidobrensis* by (Migiro *et al*., 2010). When a spore comes in contact with the insect cuticle, the spore attaches, germinates, penetrates the cuticle (from outside in, or inside

out, depending on whether the spore lands on the outer surface, or is ingested). Penetration from outside-in appears more common. Many insects avoid disease when spores are ingested.

The other strategy is the use of fungal endophytes which are heterotrophic micro-organisms that live inside plants tissues primarily for nutrition, protection and reproduction (Azevedo *et al*., 2000; Backman & Sikora, 2008). Additionally, some fungal endophytes are known to boost plant growth and activate plant defense mechanisms against various insect pests and parasitic nematodes (Sikora *et al*., 2008), thus drastically reducing damages associated with pest infestation and feeding. The endophytes are introduced to the plant through seed inoculation before sowing them. Clay (1989), in a review on the potential of insect control by endophytic fungi, stressed that insect biocontrol may be improved by the development of artificial inoculation techniques.

Recent studies carried out at ICIPE have shown the ability of some fungal pathogens to endophytically colonize bean plants and confer their resistance against *Liriomyza* in cage experiments (Akutse *et al*., 2013). *Hypocrea lixii* isolate F3ST1 and *Beauveria bassisana* isolate GILU3 were identified as the best isolates and were selected for further studies (Akutse *et al*., 2013). As long as many pesticides continue to face severe restrictions on usage and withdrawal from the markets, the demand and market opportunities for using such biological control agents (BCAs) in crop protection will continue to grow.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

The field study was conducted in two locations: Naromoru (27°23'S, 21°83'W), 2,036 meters above sea level (masl) and Sagana (35°37'S, 18°75'W), 1,208 masl, Nyeri County, Central Kenya (Figure 2.1). The annual mean temperature is 23°C (max. 26°C and min. 19°C) and annual mean rainfall is 1,700 mm, which occurs in bimodal patterns of March-July (long rain) and October- December (short rain), and are the two main growing seasons. Naromoru has black cotton soil and clay in some areas while Sagana has volcanic soil. This has led to high production of *Phaseolus vulgaris.* The two locations are also under irrigation system; hence crops are planted throughout the year leading to high infestation of *L. huidobrensis*. Farms of small-scale farmers were selected from the two locations (Naromoru and Sagana) because of their deep, fertile soil, from loose to slightly compact, with good physical properties and flat or rolling topography with good drainage where two 100 m^2 experimental fields/plots were selected in each location. The experiment was laid on a randomized complete block design (RCBD) with six treatments each replicated four times. The study was conducted for two cropping seasons May-August (long rains) and September-December (short rains). All the laboratory experiments were conducted at the International Centre of Insect Physiology and Ecology (ICIPE) in Duduville campus, Nairobi, Kenya (1° 18'S, 36° 49'E) and 1798 masl.

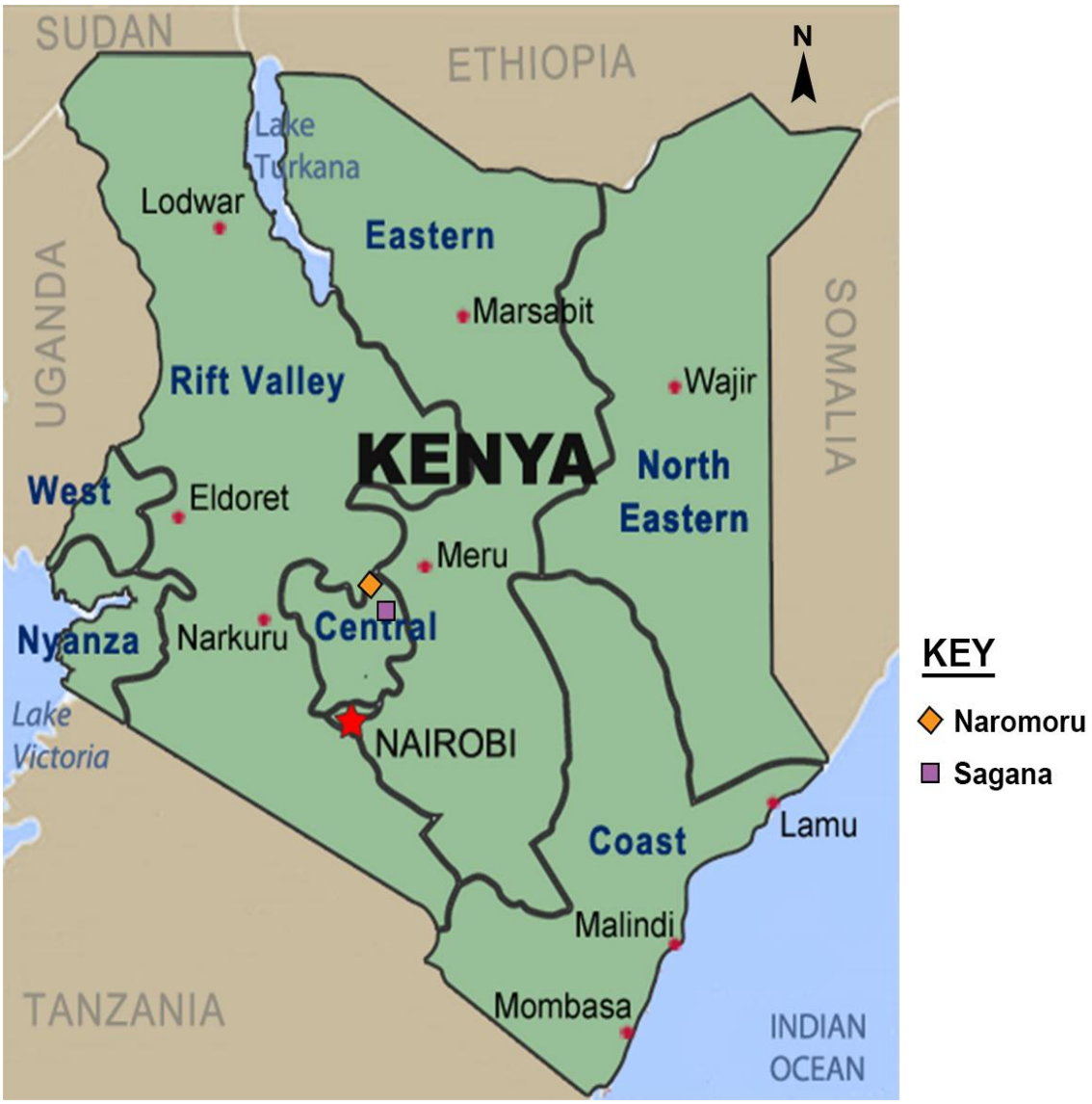

Figure 3.1: A map of Kenya showing study sites

3.2 Fungal isolates cultures

Endophytic fungi, which live within host plant tissues without causing any visible symptom of infection, are important mutualists that mediate plant–herbivore interactions. *Beauveria bassiana* isolates G1LU3 and *Hypocrea lixii* isolate F3ST1, previously reported pathogenic to *L. huidobrensis* and *M. anisopliae* known to be highly virulent to *L. huidobrensis* were used in this study. *Beauveria bassiana* isolates G1LU3 and *H. lixii* isolate F3ST1 were isolated from the aboveground parts of maize, sorghum

and Napier grass while *Metarhizium anisopliae* ICIPE 20, developed by the International Centre of Insect Physiology and Ecology (ICIPE) from *Metarhizium anisopliae*, a fungus that grows naturally in the soil.

Fungal isolates, *Hypocrea lixii* isolate F3ST1 and *Beauveria bassiana* isolate G1LU3 which were previously reported to endophytically colonize bean plants (Akutse *et al*., 2013) were obtained from the ICIPE's Arthropod Germplasm Centre. *Metarhizium anisopliae* (Metschnikoff) Sorokin (Hypocreales: Clavicipitaceae) isolate ICIPE 20 was previously reported virulent to adult *L. huidobrensis* (Migiro *et al*., 2010) and was included in the present study. *Metarhizium anisopliae* ICIPE 20 was cultured on Sabouraud Dextrose Agar (SDA) while *H. lixii* F3ST1 and *B. bassiana* G1LU3 were cultured on Potato Dextrose Agar (PDA) and the plates were 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 determined before being used for field experiments based on germ tube formation (Goettel & Inglis, 1997).

3.3 Mass production of fungal isolates

Conidia of the three isolates were mass produced on whole rice substrate in Milner bags (60 x 35 cm). Rice was autoclaved for 1 hour at 121° C and inoculated with a 3-day old culture of blastospores. Bags were incubated for 21 days at 20-26 ° C, 40-70% RH and allowed to dry for 5 days at room temperature. Conidia were harvested by sifting the substrate through a sieve (295 mm mesh size) and stored at 4-6 ° C for less than three weeks before being used. The viability of the three fungal isolates was determined before being used for field experiments according to the technique described by Goettel and Inglis (1997). Suspension (0.1 ml) titrated 3 x 10^6 conidia ml^{-1} was spread-plated onto 9-cm Petri dishes containing SDA or PDA. A sterile microscope cover slip (2 x 2 cm) was placed on the top of the agar in each plate. Plates were incubated in complete darkness at 25 ± 2 ° C and examined after 16-20 hours. The percentage germination of conidia was determined from 100 randomly selected conidia on the surface area covered

by each cover slip under the light microscope (400x). Conidia were deemed to have germinated when the length of the germ tube was at least twice the diameter of the conidium. Conidial germination of approx. 95% was observed with the three fungal isolates.

3.4 Seed inoculation

The harvested conidia were then mixed in 10 ml sterile distilled water containing 0.05% Triton X-100 and vortexed for 5 minutes to produce homogenous conidial suspensions. Triton water was added to reduce the surface tension between water and the conidial spores. Conidial counts (average spore counts) were done using a Neubauer Hemacytometer (Goettel & Inglis, 1997). The constant number of chamber used was 10^2. The conidial suspension (C_2) was adjusted to 1 x 10^8 conidia ml^{-1} through serial dilution prior to inoculation of seeds (Akutse *et al*., 2013). The stock concentration (C_1) was obtained by multiplying the average conidial count by the dilution factor (10^2) and by the Neubauer Hemacytometer constant (2.5 x 10^5).

Stock Concentration (C_1) = Average count x Dilution factor x Hemacytometer Constant

The working concentration (V_1) obtained by multiplying the conidial suspension (C_2) by the desired volume (V_2) then dividing the product by the stock concentration (C_1)

$$\text{Working concentration } (V_1) = \frac{\text{Conidial suspension } (C_2) \times \text{Desired Volume } (V_2)}{\text{Stock concentration } (C_1)}$$

Seed inoculation was done by soaking batches of approximately 220 seeds of *P. vulgaris* (Red Rose Coco, a local Kenyan open pollinated variety) per treatment in 300 ml conidial suspensions titrated 1 x 10^8 conidia ml^{-1} in 500-ml Erlenmeyer flasks for 2 hours. Prior to inoculation, seeds were surface-sterilized in 70% ethanol for 2 min followed by 1.5% sodium hypochlorite for 3 min and rinsed with sterile distilled water three times. For the controls, sterilized seeds were soaked in sterile distilled water for 2 hours. The last rinse water was plated out to assess the effectiveness of the surface

sterilization procedure (Akutse *et al*., 2013). Seeds were then planted within 24 hours in the experimental units as per above defined treatments.

3.5 Crop (*Phaseolus vulgaris*)

Phaseolus vulgaris is widely cultivated for its delicious seeds which add flavour and protein to the diets of millions of people throughout the world. Seeds of *P. vulgaris* (Red Rose Coco), a local Kenyan open pollinated variety were used for the experiment. *Phaseolus vulgaris* seeds were sown within 24 hours after their inoculation. Watering (using watering can or sprinkler) of seeds was done after every three days until germination and during scarcity of rains. Two weeks after germination, thinning was done so as to keep only two plants per hole (120 plants per treatment) and also samples from uprooted plants were used for fungal colonization test. The success rate (%) of fungal colonization of host plants parts was calculated as follows:

$$\% \text{ colonization} = \frac{\text{Number of pieces exhibiting fungal outgrowth}}{\text{Total number of pieces plated out}} \times 100\%$$

Weeding was done monthly to maintain the plots clean and also reduce pests' infestation. A hoe was used in the first weeding where as in the subsequent weeding hands were used to uproot weeds to avoid breaking the bean plants due to high vegetation at the canopy.

3.6 Experimental design and treatments

Field trials were established in the farms of small-scale farmers in the study sites (Sagana and Naromoru). Two 100m^2-experimental plots were selected in each location. Each 100 m^2 plot was divided into twelve experimental units measuring 3 m^2 (2 m x 1.5 m) each, where three treatments with four replicates which were randomly assigned. Each experimental unit comprised of 60 planting holes (2 – 3 cm depth) where spacing between rows was 40 cm and 15 cm within the plants. Three seeds (inoculated and non-inoculated) were sown per hole (2–3 cm depth) and amended with 250 grams of manure.

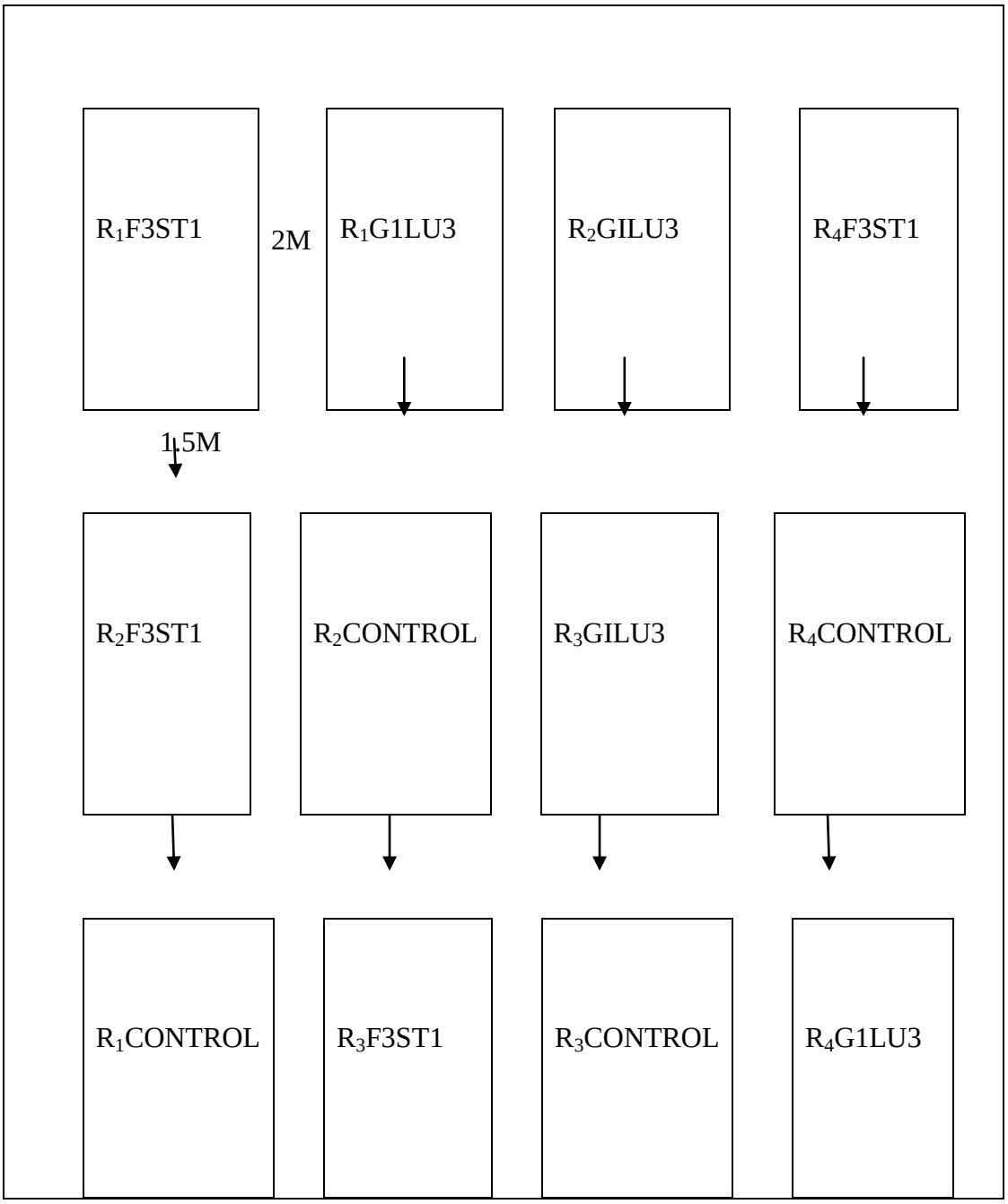

Figure 3.2: Sampled treatments in the experimental units in a field layout

At each location, two fields were planted with both fungal inoculated seeds and uninoculated seeds. Autodissemination devices (AD) which were made locally and had the ability to attract and infect leafminer flies were included in one of the plots. The

following treatments were applied: (T1) *B. bassiana* G1LU3-inoculated bean seeds, (T2) *H. lixii* F3ST1-inoculated bean seeds, (T3) *B. bassiana* G1LU3-inoculated bean seeds + *M. anisopliae* ICIPE 20 applied in autodissemination device (AD) (*B. bassiana* G1LU3 + AD), (T4) *H. lixii* F3ST1-inoculated bean seeds + *M. anisopliae* ICIPE 20 applied in autodissemination device (*H. lixii* F3ST1 + AD), (T5) uninoculated seeds + *M. anisopliae* ICIPE 20 applied in autodissemination device (control + AD) and (T6) uninoculated seeds without autodissemination device (control).

Figure 3.3: Field 1 planted with both fungal inoculated seeds and uninoculated seeds in the absence of autodissemination device

Autodissemination devices were hanged 30 cm above the ground at the middle of a string supported by two strong sticks, and the height of the string was fortnightly adjusted depending on the height of the bean plants as they grew (Figure 3.4). Treatments were randomized in complete block design (RCBD) with four replicates per season and all the experiment was repeated in two consecutive seasons. Note that "fungus or endophyte treatments" and "endophytically-inoculated" was used interchangeably.

Figure 3.4: Field 2 planted with both fungal inoculated seeds and uninoculated seeds in the presence of autodissemination device

3.7 Autodissemination device

The autodissemination device used in the present study is the one described by Migiro *et al*., (2010) which was selected and evaluated for its ability to attract and infect flies. The device made is cost-effective because materials used are locally available, and the device can be produced locally. The device was made of a 500-ml clear disposable plastic beverage bottles (5.5 cm diameter, 21 cm in height). 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. A dark grey cotton velvet cloth (8 cm diameter × 8.5 cm length) was used to hold the dry conidia of *M. anisopliae* ICIPE 20. It was held in place by gluing it on its smooth side and wrapping it around a smaller inner cylindrical bottle (5.2 cm diameter, and 6 cm in height) exposing the rough surface. A yellow netting (8.9 cm diameter × 8.6 cm length) of 1 mm^2 holes was wrapped around the velvet cloth and tightened with two office pins to attract flies. The smaller plastic diameter bottle was later introduced into the bigger bottle and fixed at the center using a metallic wire which serves for hanging

the device. On the velvet wrapping approximately 3 grams of dry conidia (in powder form) of *M. anisopliae* ICIPE 20 were applied per trap before introducing it in the field (Figure 3.5). The *M. anisopliae* ICIPE 20 conidia were boosted once per season (six weeks after introduction of the devices).

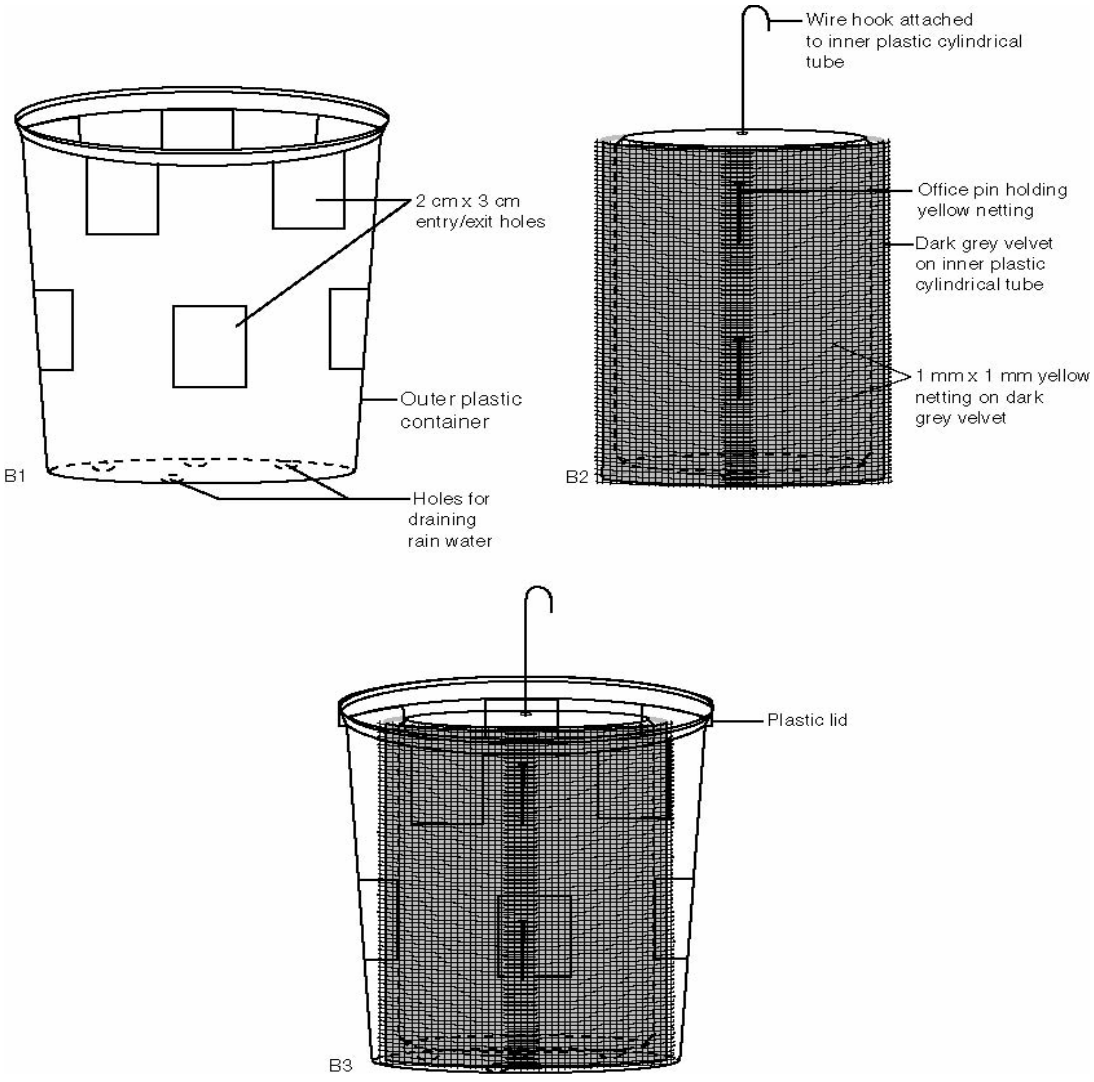

Figure 3.5: The autodissemination device used to apply *M. anisopilae* ICIPE 20

(Migiro *et al*., 2010)

3.8 Evaluation of treatments

3.8.1 Endophytic colonization of *Phaseolus vulgaris* host plants

Three weeks after sowing, seedlings were sampled at random for endophytic colonization assessment. Seedlings in fungus-inoculated seeds were carefully uprooted and brought to the laboratory. They were washed with tap water and cut into separate different sections (leaves, stems and roots sections) of 2 cm long pieces. The latter were surface-sterilized in 70% ethanol for 2 min followed by 1.5% sodium hypochlorite for 3 min and rinsed with sterile distilled water three times.

The last rinse water was plated out to assess the effectiveness of the surface sterilization procedure (Akutse *et al*., 2013). This was followed by aseptically cutting them under a laminar flow hood into 1 x 1 cm pieces. Five pieces of each section were sampled and placed 3 cm apart on PDA plates amended with a 0.05% solution of antibiotic (streptomycin sulfate salt) (Dingle & McGee, 2003; Istifadah & McGee, 2006; Gurulingappa *et al*., 2010; Akutse *et al*., 2013). Plates were incubated at 25 ± 1°C for 10 days, after which the presence of endophytes was determined. The last rinse water was also plated out on PDA medium to assess the effectiveness of the surface sterilization procedure as described earlier. Colonization of the different plant parts were recorded by counting the number of pieces of the different plant parts that showed the presence of inoculated fungal growth/mycelia according to Koch's postulates (Petrini & Fisher, 1987). Only the presence of endophytes that were inoculated and successfully colonized the different plated plant parts were scored.

3.8.2 *Liriomyza* leafminer flies and parasitoids population densities

Biweekly samplings of the crops were conducted to determine LMF and parasitoids species abundance and density from 18th June, 2013 until 11th September, 2013 in the long rains season and from 9th October, 2013 until 4th December, 2013 in the short rains season in the two experimental sites. To determine the density of LMF and their associated parasitoids, each experimental unit of 3 m^2 was subdivided into four equal

quadrants (0.75 m^2 each) and from each quadrant the number of adult LMF and parasitoids present per m^2 on the canopy were directly counted and recorded. Care was taken not to interfere with the leaves to avoid insects flying away (although LMF are not strong flyers) or into other quadrants. Samples of LMF and parasitoids were brought to the laboratory for identification.

3.8.3 *Liriomyza* leafminer flies infestation

Two types of sampling were carried out biweekly to determine LMF infestation. First, each experimental unit was sub-divided into four quadrants from which 25 leaves (giving a total of 100 leaves per treatment) were picked at random (from the middle stratum of different plants) and examined for leafminer punctures and/or mines. The total numbers of infested leaves were recorded. During the first six weeks after germination, the 25 leaves per quadrant were marked with a mark pen to avoid sampling the same leaves many times. This was done to avoid destroying leaves at the early stage of plant development to maximize photosynthesis and avoid stress of the young seedlings. A different color of mark pen was used in the consecutive samplings for easy identification.

The second type of sampling consisted of collecting a maximum of 50 infested leaves with developing or developed LMF mines containing live leafminer larvae per experimental unit through active searching. The infested leaves were immediately placed in perforated plastic paper bags, labeled and transferred into plastic rearing containers /lunch boxes (19 cm ×13 cm × 8 cm) lined with paper towels to absorb excess leaf moisture and also to avoid drying of leaves. The rearing containers were closed with lids containing muslin (16 cm × 9.5 cm) for ventilation and were parked in large cool boxes to avoid overheating before transportation to the laboratory where they were maintained at 25 ± 2°C and 60 ± 5% R.H. After 5-10 days, pupae were collected from the rearing containers using soft camel hair brushes and incubated in open plastic Petri dishes in Perspex cages until adults of leafminer flies and parasitoids emerged. Adult *Liriomyza* species as well as their associated parasitoids were collected, counted and preserved in

80% ethanol and identification done using conventional taxonomic keys and identification keys from the *Liriomyza* leafminer flies project at the International Center of Insect Physiology and Ecology (*ICIPE*), Duduville campus, Nairobi, Kenya. Voucher specimens of identified adult *Liriomyza* species and parasitoids were stored in the entomological museum at *ICIPE*.

3.8.4 *Phaseolus vulgaris* yield assessment

Harvesting of *Phaseolus vulgaris* was done after the leaves of the *P. vulgaris* plants had dried and most of them fallen on the ground hence exposing the pods which had changed their colour from green to brown and some curling signifying dryness. Harvesting was done on week 16 and 19 post-germination at Sagana and Naromoru, respectively, for the first season while on week 13 at Sagana and week 17 post-germination at Naromoru in the second season. The number of plants and pods per plant per treatment was counted and recorded before uprooting. Ten pods were randomly selected and the number of seeds per pod was counted and recorded. To assess the yield, hand threshing of dried seeds was done before weighing the total seeds harvested from each treatment. The yield weight per treatment was then converted and extrapolated into tonnes per hectare. The actual moisture content of the seeds was assessed before weighing using Instant Moisture Meter Kett PM-600 (Kett Electric Laboratory 1-8-1 Minami-Magone Ota-Ku, Tokyo, Japan) (Figure 3.6).

Figure 3.6 Assessment of moisture content and weighing of the seeds for yield determination

3.9 Data analysis

Fungal colonization percentages were arcsine-transformed before subjecting to analysis of variance (ANOVA). Data were analyzed separately for each site according to the season and the host plant parts but comparison was done among the various treatments. Data on LMF-infested leaves were recorded as grouped binary data and analyzed using a generalized linear model (GLM) assuming a binomial distribution error and logit link. Since leaves were sampled over time, to avoid pseudo-replication, the percentage of LMF-infested leaves from each replicate was calculated as number of infested leaves divided by total number of leaves sampled multiplied by hundred and averaged over time and used in analysis (Hurlbert, 1984). Biweekly pupae recovered and LMF abundance data were averaged over the weeks and analyzed using a generalized linear model (GLM) assuming a negative binomial distribution error and logarithmic link. In cases of over-dispersion, the negative binomial distribution error was assumed for the pupae counts (O'Hara & Kotze, 2011). The effect of treatment factors in a GLM is reflected in the deviance that has an approximate chi-square distribution; hence, the chi-

square values are presented as test statistics. Biweekly emerged parasitoids from LMF-infested leaves were summed and analysed using a GLM, as was the case with the LMF and pupae counts. Adjusted Tukey method was used for the GLM analysis to separate treatment means implemented using the *multcomp* package in R (Hothorn *et al*., 2008). Yield data were analyzed using ANOVA and means were separated using Student Newman Keuls (SNK) test. All data were analyzed in R version 3.0.2 statistical software (R Development Core Team 2013).

CHAPTER FOUR

RESULTS

4.1 Endophytic colonization of *Phaseolus vulgaris* host plant parts

The two isolates (*B. bassiana* G1LU3 and *H. lixii* F3ST1) colonized all the three parts (roots, stems and leaves) of the *P. vulgaris* host plant while their colonization was absent (no growth) in the control plants. There were no significant interaction effects (χ^2 = 0.03; df = 2; P = 0.8561) between sites and fungal treatments in colonization of roots, stems and leaves. However, there were significant differences between the two isolates and the host plant parts when comparing the treatments among themselves. For example, at Naromoru in season 1 (long rains season), significant differences in colonization by isolates were observed on roots ($F_{3,12}$ = 38.00; P < 0.0001) where *H. lixii* F3ST1 colonized 95–100 % and *B. bassiana* G1LU3 30–35 %, on stems ($F_{3,12}$ = 7.02; P = 0.006) where *H. lixii* F3ST1 colonized about 95 % and *B. bassiana* G1LU3 45–50 %, and on leaves ($F_{3,12}$ = 22.30; P < 0.0001) where *H. lixii* F3ST1 colonization was 100 % compared to 55–65 % for *B. bassiana* G1LU3 (Figure 4.1). In the same season 1 (long rains season) at Sagana, significant differences in colonization between isolates were observed on roots ($F_{3,12}$ = 23.75; P < 0.0001) with *H. lixii* F3ST1 colonizing between 95–100 % and *B. bassiana* G1LU3 40–50 %, on stems ($F_{3,12}$ = 29.27; P < 0.0001) with 95–100 % colonization by *H. lixii* F3ST1 and 35–40 % by *B. bassiana* G1LU3, and on leaves ($F_{3,12}$ = 6.38; P = 0.008) where *H. lixii* F3ST1 colonized 100 and 50 % for *B. bassiana* G1LU3 (Figure 4.1).

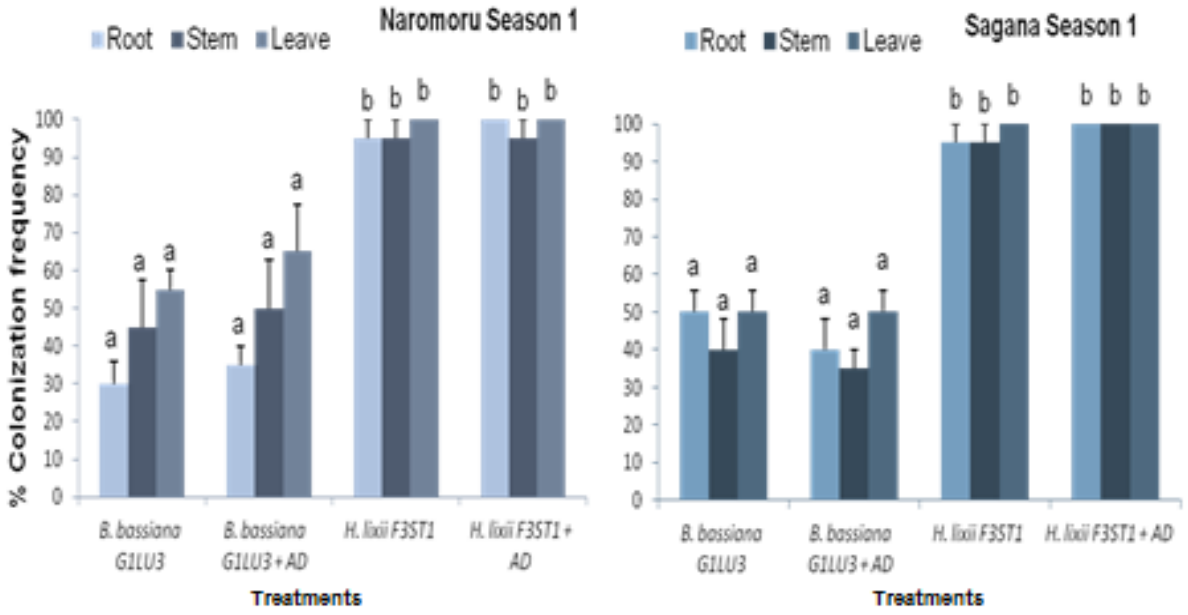

Figure 4.1: Endophytic mean colonization frequency of different parts of *P. vulgaris* plant in the field during season 1 (long rains season) at Naromoru and Sagana

AD autodissemination device. Bars are means ± SE. Mean separations were done for each plant part (root, stem and leave). Means followed by the same letter for each plant part are not significantly different at $P < 0.05$ by Tukey's test.

Similar trends were observed in season 2 (short rains season) at Naromoru with significant differences in colonization by isolates on roots ($F_{3,12} = 13.53$; $P = 0.0004$) where *H. lixii* F3ST1 colonization was about 95 % compared to 40–45 % for *B. bassiana* G1LU3, on stems ($F_{3,12} = 4.82$; $P = 0.020$) where *H. lixii* F3ST1 colonized about 85 % of the stems compared to 40 % for *B. bassiana* G1LU3, and on leaves ($F_{3,12} = 29.12$; $P < 0.0001$) with 100 % colonization by *H. lixii* F3ST1 compared to 55–60 % for *B. bassiana* G1LU3 (Figure 4.2).

Similar trends were observed in the season 2 (short rains season) at Sagana with significant differences in colonization by isolates on roots ($F_{3,12} = 13.53$; $P = 0.0004$) where *H. lixii* F3ST1 colonized about 95 % of the roots compared to 40–45 % for *B.*

bassiana G1LU3, on stems ($F_{3,12}$ = 7.77; P = 0.004) with 90 % colonization by *H. lixii* F3ST1 and 40 % by *B. bassiana* G1LU3, and on leaves ($F_{3,12}$ = 50.97; P < 0.0001) with 100 % colonization by *H. lixii* F3ST1 compared to 55–60 % for *B. bassiana* G1LU3 (Figure 4.2). No differences in the host plant colonization ($F_{3,12}$ = 1.89; P = 0.1867) were observed in presence or absence of *M. anisopliae* ICIPE 20 autodissemination traps in the field (Figure 4.2).

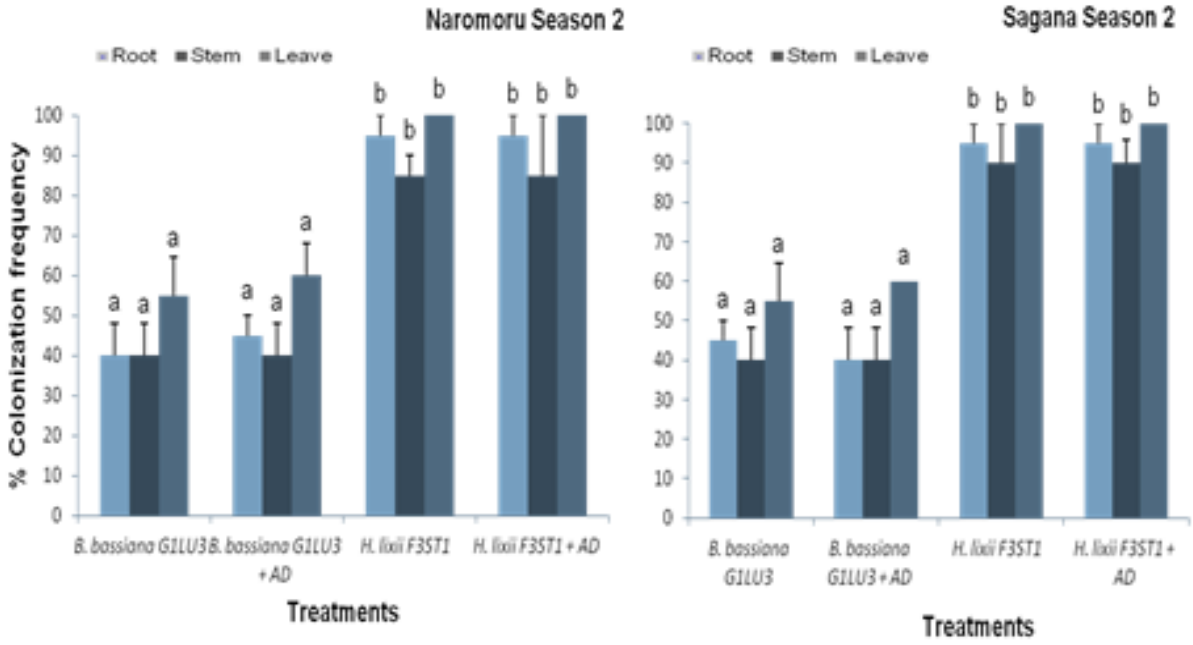

Figure 4.2: Endophytic mean colonization frequency of different parts of *P. vulgaris* plant in the field during season 2 (short rains season) at Naromoru and Sagana

AD autodissemination device. Bars are means ± SE. Mean separations were done for each plant part (root, stem and leave). Means followed by the same letter for each plant part are not significantly different at P < 0.05 by Tukey's test.

4.2 *Liriomyza* leafminers infestation level

Leafminer infestation was higher during the season 1 (long rains season) compared to season 2 (short rains season) but there was no significant differences between the two study sites (χ^2 = 0.21; df = 2; P = 0.89). No significant interactions were observed between sites and treatments as regard to infestation levels in the long rain season (χ^2 = 0.748, df = 5, P = 0.98). Considering the treatments at each site in the season 1 (long rains season), there were no differences in the treatments at Naromoru (χ^2 = 9.53, df = 5, P = 0.09) and Sagana (χ^2 = 6.00, df = 5, P = 0.31) (Figure 4.3).

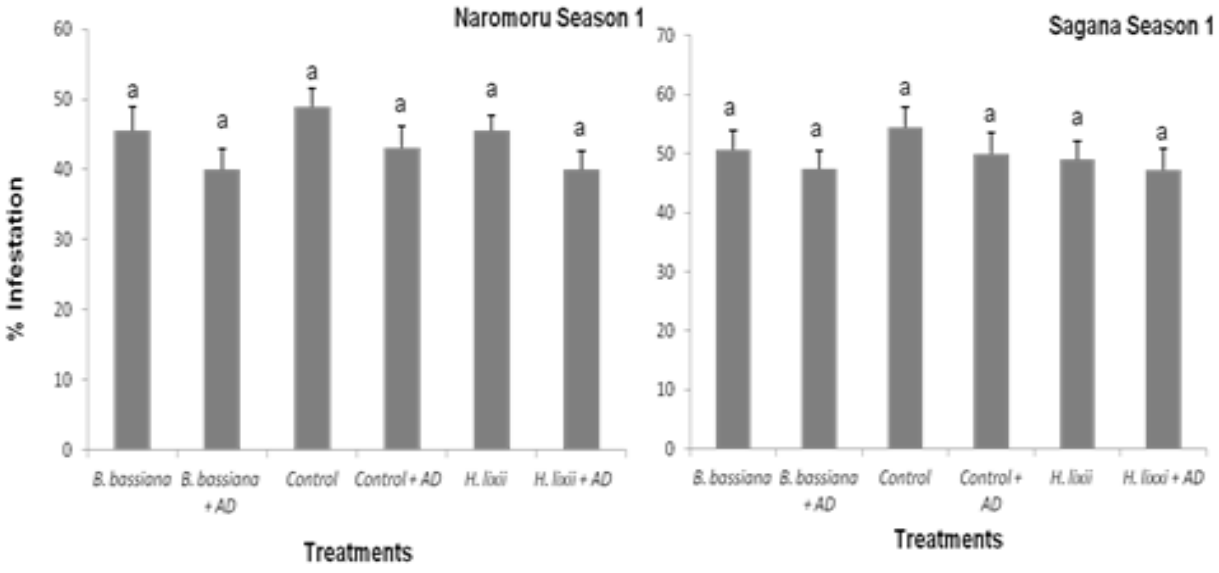

Figure 4.3: Leafminer fly infestation level as regards to the treatments at Naromoru and Sagana during season 1 (long rains season)

AD autodissemination device. Bars are means ± SE. Means followed by the same letter are not significantly different at P < 0.05 by Tukey's test.

However, in the season 2 (short rains season), there were significant interactions between sites and treatments (χ^2 = 20.25, df = 5, P = 0.001) in LMF infestation levels.

Subsequently, treatments at each site were significantly different at Naromoru (χ^2 = 132.47, df = 5, P < 0.0001) and Sagana (χ^2 = 103.46, df = 5, P <0.0001) (Figure 4.4). Endophytically-colonized *P. vulgaris* plants had lower infestation levels compared to the controls in both sites in season 2 (short rains season) (Figure 4.4). The addition of *M. anisopliae* ICIPE 20 in autodissemination devices did not result in significant differences between the treatments, except in the control at Naromoru and *H. lixii* F3ST1 inoculated bean plants in Sagana during the second season (short rains season) (Figure 4.4).

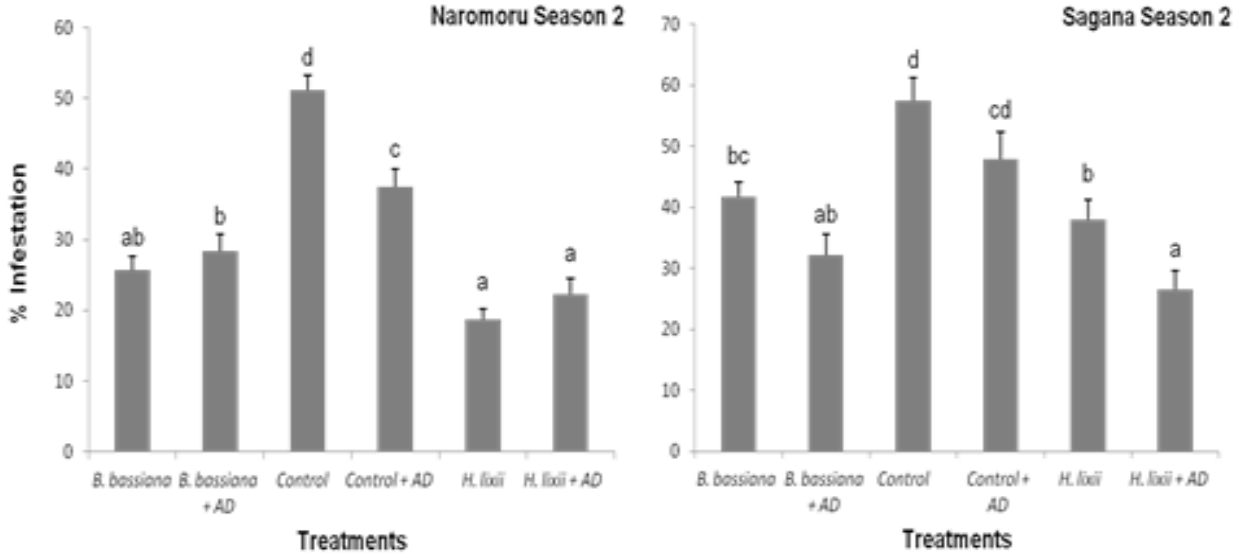

Figure 4.4: Leafminer fly infestation level as regards to the treatments at Naromoru and Sagana during season 2 (short rains season)

AD autodissemination device. Bars are means ± SE. Means followed by the same letter are not significantly different at P < 0.05 by Tukey's test.

4.3 *Liriomyza* leafminers and their associated parasitoids population densities

Three key *Liriomyza* species (*L. huidobrensis*, *L. sativae* and *L. trifolii*) and six parasitoid species (*Opius dissitus* Muesebeck, *Phaedrotoma scabriventris* Nixon (Hymenoptera: Braconidae), *Diglyphus isaea* Walker, *Neochrysocharis formosa* (Westwood), *Hemiptarsenus varicornis* (Girault) (Hymenoptera: Eulophidae) and *Halticoptera arduine* Walker (Hymenoptera: Pteromalidae) were identified during the surveys. *Liriomyza huidobrensis* were the most abundant LMF species with more than 90% of all LMF collected species while *D. isaea* was the most abundant among the parasitoids accounting for 69% of the total parasitoids recorded. There was no interaction effects between LMF ($\chi^2 = 0.34$, df = 2, P = 0.91) and parasitoid ($\chi^2 = 2.89$; df = 2; P = 0.27) densities as regards to the locations and seasons. For *Liriomyza* leafminers, no significant differences ($F_{2, 22} = 1.62$; P = 0.23) were observed between the treatments during long and short rains seasons regardless of the field sites. The overall mean of LMF density in the long rains season (season 1) varied between 4.0 ± 0.6 and 5.3 ± 0.8 and between 5.0 ± 0.2 and 8.7 ± 4.5 LMF m^{-2} at Naromoru and Sagana, respectively (Table 4.1). During the short rains season (season 2), the overall mean LMF density ranged between 0.9 ± 0.2 and 1.4 ± 0.2 and between 3.2 ± 1.4 and 4.6 ± 1.1 LMF m^{-2} at Naromoru and Sagana, respectively (Table 4.1).

Table 4.1: Mean number of leafminer fly m^{-2} (± SE) collected during the long rains and short rains seasons in Naromoru and Sagana (n = 3)

Treatments	Mean number of LMF m^{-2} (± SE) per season and locality			
	Long rains season		Short rains season	
	Naromoru	Sagana	Naromoru	Sagana
Beauveria bassiana	4.3 ± 1.4	7.9 ± 3.9	1.1 ± 0.4	4.6 ± 1.1
Beauveria bassiana + AD	5.0 ± 1.0	6.1 ± 0.6	0.9 ± 0.2	3.3 ± 1.3
Hypocrea lixii	4.0 ± 0.6	8.7 ± 4.5	1.3 ± 0.4	4.6 ± 1.2
Hypocrea lixii + AD	4.7 ± 0.7	5.0 ± 0.2	1.3 ± 0.3	4.1 ± 1.7
Control	4.9 ± 1.0	7.8 ± 3.8	1.4 ± 0.2	4.4 ± 1.2
Control + AD	5.3 ± 0.8	5.5 ± 0.7	1.0 ± 0.2	3.2 ± 1.4
Df	5160	5162	5114	5114
F value	0.5	0.8	0.8	0.9
P value	0.1202	0.3108	0.5261	0.4738

AD autodissemination device

The mean numbers of parasitoids that emerged from pupae collected from the 50 infested leaves with developing or developed LMF mines containing live leafminer larvae per treatment were higher during the long rains season (season 1) and varied between 13.9-26.1 and between 16.7-22.1 parasitoids at Naromoru and Sagana, respectively, than during the short rains season (season 2) where they varied between 3.4-6.6 and between 6.2-12.5 parasitoids at Naromoru and Sagana, respectively. However, the number did not differ significantly among the treatments at Naromoru ($F_{5,160}$ = 0.5; P = 0.1202 and $F_{5,114}$ = 0.8; P = 0.5261) and Sagana ($F_{5,162}$ = 0.8; P = 0. 3108 and $F_{5,114}$ = 0.9; P = 0. 4738) in both seasons.

4.4 Effects of fungal treatments on leafminer pupae

There was significant interaction between sites and treatments in season 1 (long rains season) (χ^2 = 11.77, df = 5, P = 0.04), while there was none in season 2 (short rains season) (χ^2 = 1.96, df = 5, P = 0.86). More pupae were collected in the controls than in endophyte treatments during the season 1 (long rains season) at Naromoru (χ^2 = 669.17, df = 5, P < 0.0001) and Sagana (χ^2 = 123.78, df = 5, P < 0.0001). Similar results were obtained in the season 2 (short rains season), where significant differences were observed between the treatments at Naromoru (P < 0.0001) and Sagana (P < 0.0001). The number of pupae varied between 141 and 252 and between 331 and 416 in endophyte and control treatments, respectively, during season 1 (long rains season) (Figure 4.5) and from 110 to 223 and from 366 to 523, respectively, in endophyte and control treatments during season 2 (short rains season) (Figure 4.6). During season 1 (long rains season), few numbers of pupae were collected in *H. lixii* F3ST1 (144.9±1.4) and *B. bassiana* G1LU3 (251.6 ± 2.2) compared to the control (410.7 ± 1.3) at Naromoru versus 141.0±1.9 (*H. lixii* F3ST1) and 199.3 ± 1.9 (*B. bassiana* G1LU3) compared to the control (415.9 ± 2.7) at Sagana (Figure 4.5). In season 2 (short rains season), fewer pupae were obtained in *H. lixii* F3ST1 (112.9 ± 2.8) and *B. bassiana* G1LU3 (219.1 ± 3.4) compared to the control (509.3 ± 4.0) at Naromoru versus 109.9 ± 2.3 (*H. lixii* F3ST1) and 222.8 ± 4.1 (*B. bassiana* G1LU3) compared to the control (523.0 ± 8.3) at Sagana (Figure 4.6). However, *B. bassiana* G1LU3 endophyte treatment did not differ significantly from control + autodissemination device (Control + AD) in the short rains season (season 1) at both sites for *Liromyza* leafminer infestation levels (Figure 4.6).

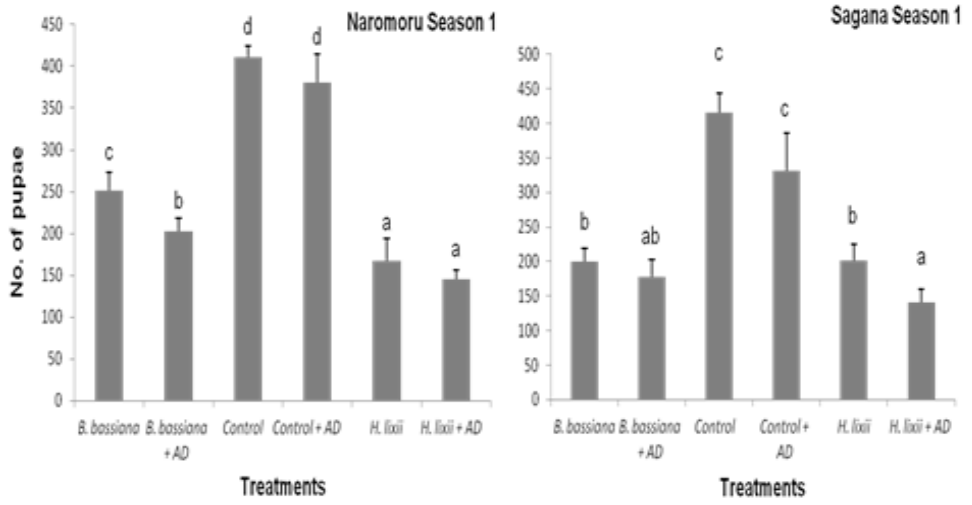

Figure 4.5: Number of pupae per treatment and per plot/field collected from infested leaves at Naromoru and Sagana during the long rains season (season 1)

AD autodissemination device. Bars are means ± SE. Means followed by the same letter are not significantly different at $P < 0.05$ by Tukey's test.

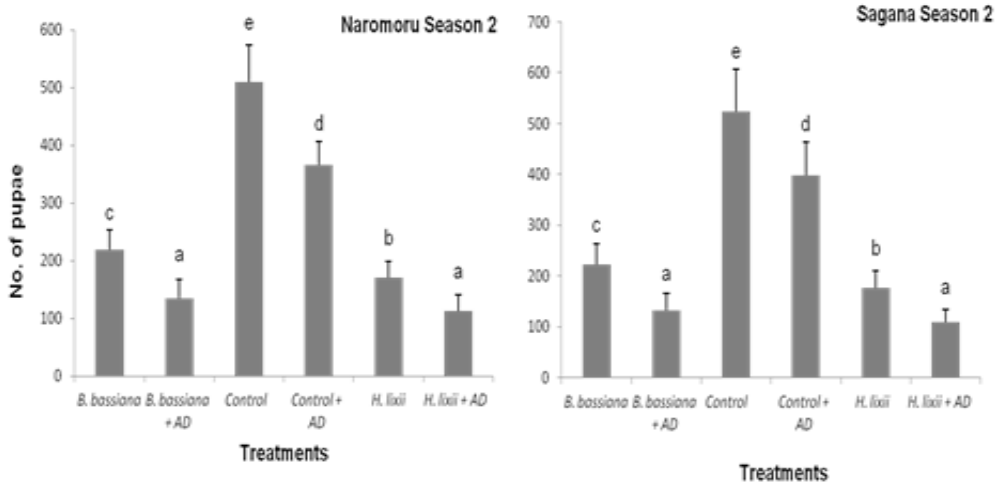

Figure 4.6: Number of pupae per treatment and per plot/field collected from infested leaves at Naromoru and Sagana during the short rains season (season 2)

AD autodissemination device. Bars are means ± SE. Means followed by the same letter are not significantly different at $P < 0.05$ by Tukey's test.

4.5 *Phaseolus vulgaris* seed yield

There were significant differences in *P. vulgaris* seed yield between the treatments in the long rains season (season 1) at Naromoru ($F_{5,18} = 14.37$; $P < 0.0001$), Sagana ($F_{5,18} = 41.23$; $P < 0.0001$) and the short rains season (season 2) at Naromoru ($F_{5,18} = 35.03$; $P < 0.0001$) and at Sagana ($F_{5,18} = 47.19$; $P < 0.0001$). Higher yields were obtained in fungal treatments as compared to the control (Table 4.2). However, the yields varied among the fungal treatments. For instance, yield was significantly higher in *H. lixii* F3ST1 + AD treatment during the long rain season (season 1) at Sagana and both sites during the short rains season (season 2) than in *B. bassiana* G1LU3 treatment (Table 4.2).

Table 4.2: Calculated mean yield of *Phaseolus vulgaris* at Naromoru and Sagana during the long rains and short rains seasons

Treatments	**Yield (t ha^{-1}) (± SE) per season and locality**			
	Long rains season		**Short rains season**	
	Naromoru	**Sagana**	**Naromoru**	**Sagana**
B. bassiana G1LU3	4.9 ± 0.3 bc	4.9 ± 0.2 c	3.6 ± 0.1 bc	3.6 ± 0.1 b
B. bassiana G1LU3 + AD	5.4 ± 0.1 bc	5.4 ± 0.0 cd	4.0 ± 0.1 c	3.9 ± 0.1 bc
H. lixii F3ST1	5.6 ± 0.2 bc	5.4 ± 0.2 cd	3.9 ± 0.1 c	4.1 ± 0.1 c
H. lixii F3ST1 + AD	5.8 ± 0.2 c	5. 8 ± 0.1 d	4.5 ± 0.1 d	4.5 ± 0.0 d
Control	3.7 ± 0.2 a	3.5 ± 0.0 a	2.2 ± 0.5 a	2.4 ± 0.2 a
Control + AD	4.8 ± 0.0 b	4.2 ± 0.1 b	3.0 ± 0.3 b	2.7 ± 0.1a
Df	518	518	518	518
F value	14.37	41.23	35.03	47.19
P value	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means followed by the same letter in rows and columns are not significantly different at P = 0.05 by Student Newman Keuls (SNK) test (n = 6). *AD* autodissemination device

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Entomopathogenic fungi are currently known to play multiple roles in nature such as plant endophytes, plant disease antagonists, rhizosphere colonizers, and plant growth promoters (Vega *et al*., 2009). The present study, therefore, explores the potential of fungal endophytes for the control of *Liriomyza* leafminer flies. The two fungal isolates (*B. bassiana* G1LU3 and *H. lixii* F3ST1) were able to colonize the three parts (roots, stems and leaves) of *P. vulgaris* host plant at both field sites as previously reported by Akutse *et al*., (2013) in a screen house. Similar results were reported by Akello (2012) and Duarte (2016) where *B. bassiana* endophytically colonized all the different parts of *P. vulgaris* and *Vicia faba* L. (Fabales: Fabaceae) plants. Akello (2012) also reported that *B. bassiana* S4SU1, *G. moniliformis* E3RF20, *T. asperellum* M2RT4 and *M. anisopliae* S4ST7 were able to colonize maize and bean plants. Other results were reported by Gurulingappa *et al*., (2010) with *B. bassiana* and *A. parasiticus. B. bassiana* was able to colonize entire wheat plants but not cotton, while *A. parasiticus* colonized different plant parts of wheat and cotton. However, in this study, *H. lixii* F3ST1 colonized the bean plants better than *B. bassiana* G1LU3 at Naromoru and Sagana during the long and short rains seasons.

Although not recorded, it was observed that bean plants colonized by both *B. bassiana* G1LU3 and *H. lixii* F3ST1 had higher growth rate and were more vigorous as compared to the controls, which is in agreement with El-Deeb *et al*., (2012) and Hamayun *et al*., (2012) who reported that some endophytes could enhance plant's growth through different mechanisms.

It was also observed that pods in the control treatments matured approximately two weeks earlier than the ones in the endophytically-colonized plants regardless of the location and season. Since fungal endophytes may assist their host plants for adaptation to habitat, protection against biotic and abiotic stresses, plant growth promotion or soil

nutrients uptake (Kharwar *et al*., 2008, Berg, 2009; Gond *et al*., 2010; Pandey *et al*., 2011; Li *et al*., 2012), the high yield obtained in endophytically-colonized plants could be a consequence of this growth promotion and stimulation of soil nutrients uptake in fungal treatments compared to the controls.

The variation in colonization of the different parts of *P. vulgaris* did not affect the ability of endophytes to confer protection against LMF as reported by other workers. For example, despite poor colonization of the different parts of *P. vulgaris*, isolates of *B. bassiana* ICIPE 279 and S4SU1 still had negative effects on the number of pupae and emergence of *L. huidobrensis* (Akutse *et al*., 2013). On the other hand, isolates of *M. anisopliae* ICIPE 20 were not able to colonize bean plants but were able to reduce bean stem maggot *Ophiomyia phaseoli* Tryon (Diptera: Agromyzidae) feeding, oviposition, pupation, and emergence (Mutune *et al*., 2016).

Entomopathogenic fungi as endophytes have been known to slow down reproduction rate (longer time of reproduction and fertility), reduce feeding, growth and fecundity of insects (Gurulingappa *et al*., 2010). In the long rains (season 1), LMF infestation did not vary among the treatments in both experimental sites, meaning that leafminer flies can infest both endophytically-inoculated and non-inoculated bean plants. However, it was observed that plants in the control died while endophytically-inoculated plants were able to recover and produce pods. In addition, despite many punctures (feeding and oviposition) on leaf surfaces, only few mines of the larvae developed up to pupal stage in the endophytically-inoculated treatments, subsequently resulting in lower numbers of pupae as compared to the controls. Fewer pupae were collected from *H. lixii* F3ST1 than *B. bassiana* G1LU3 treatment, which confirms the finding of Akutse *et al*., (2013) where *H. lixii* F3ST1 outperformed the other fungal isolates in reducing *Liriomyza* leafminers' progeny longevity, number of pupae, adult emergence and survival. Zhang (2014) also reported that endophytically-colonized faba bean and cabbage plants had an influence on the development and feeding behavior of the diamondback moth *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). *Plutella xylostella* larvae feeding on detached *B. bassiana*-

inoculated leaves had a slower development and a lower body weight than those fed on the control leaves (non-inoculated leaves). Similarly, Gurulingappa *et al*. (2010) reported that wheat leaves colonized by either *B. bassiana* or *Aspergillus parasiticus* Speare (Eurotiales: Trichocomaceae) reduced the growth rate of *Chortoicetes terminifera* (Walker) (Orthoptera: Acrididae) nymphs. Endophytic colonization of *B. bassiana* in banana significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* Chevrolat (Coleoptera: Curculionidae), resulting in 42-87 % reduction in plant damage (Akello *et al*., 2008).

In addition, the potential of entomopathogenic fungi for controlling insect pests has been demonstrated (Ferron *et al*., 1991; Inglis *et al*., 2001). Application of *M. anisopliae* ICIPE 20 in autodissemination devices along with endophyte treatments did affect the infestation level of *Liriomyza* leafminer flies, except with *B. bassiana* G1LU3 inoculated bean plants during the second season (short rains season) at both experimental sites. This technique has already been tested with success against tsetse flies (Maniania *et al*., 2006), fruit flies (Ekesi *et al*., 2007) and *Liriomyza* leafminers (Migiro *et al*., 2010) and could be integrated with other control strategies. It has been reported that fungal infection by mitosporic entomopathogenic fungi can affect feeding, oviposition and development of insects (Thomas *et al*., 1997; Ekesi and Maniania, 2000; Ondiaka *et al*., 2008).

Lack of significant difference in the number of parasitoids between fungus treatments and controls suggests the compatibility between fungal endophytes; *B. bassiana*, G1LU3 and *H.lixii*, F3ST1 and autodissemination of conidia of *M. anisopliae*, ICIPE 20 with parasitoids strategy in management of *Liriomyza* LMF in *P. vulgaris* under field conditions. Studying the interactions between *L. huidobrensis*, the endophytic fungi *H. lixii* F3ST1 and *B. bassiana* G1LU3, and two leafminers' parasitoid species, *Diglyphus isaea* (Hymenoptera: Eulophidae) and *Phaedrotoma scabriventris* (Hymenoptera: Braconidae) under laboratory conditions, Akutse *et al*. (2014) observed no significant difference between endophytes-free and endophyte-inoculated *Vicia faba* bean plants in

terms of parasitism rates of the two parasitoids (*P. scabriventris* and *D. isaea*) and adult survival times of both parasitoids. On the other hand, tritrophic interactions between parasitoids and host insect may vary depending on the biological attributes of the insects' relationship and the type of plant upon which they occur (Kennedy, 2003). For instance, Bixby-Brosi and Potter (2012) observed difference in endophyte-mediated tritrophic interactions among two different species of parasitoids of *Agrotis ipsilon* H. (Lepidoptera: Noctuidae). Since both *B. bassiana* G1LU3 and *H. lixii* F3ST1 were compatible with fungal endophytes, they can therefore be used in combination with LMF associated parasitoids to suppress the population of the invasive *Liriomyza* leafminers.

Higher yield was obtained in endophyte than in control treatments which could be due to the high LMF infestation in the controls, resulting in reduced photosynthetic ability. Fungal endophytes are known to boost plant growth and activate plant defense mechanisms against various insect pests (Sikora *et al*., 2008), thus drastically reducing damages associated with pest infestation and feeding which leads to higher yields. Yield average of *P. vulgaris* in Kenya is ranged between 750 kg/ha (0.75 t/ha) and 1500 kg/ha (1.5 t/ha) depending on the variety and the soil fertility (Otsyula *et al*., 1998). It has been reported that the major constraints to bean production include diseases, soil fertility, insect pests and low erratic rainfall (Otsyula *et al*., 1998). In the present study, endophyte-colonized bean plants were able to give a calculated mean yield of over 3 t/ha in the absence of autodissemination devices and over 4 t/ha in the presence of autodissemination. Autodissemination devices have been developed, whereby insects are used to vectorinoculum among conspecifics in the environment after they have been attracted and acquired the pathogen (Vega *et al*., 2007; Migiro *et al*., 2010). This leads to the ability to attract and infect flies. Hence, the incorporation of fungal endophytes *H. lixii* F3ST1 and *B. bassiana* G1LU3 in *P. vulgaris* production system may improve the management of *Liriomyza* leafminers and increase significantly the crop yield.

Fungal endophytes in combination with *M. anisopliae* ICIPE 20 applied in autodissemination device and parasitoids can contribute to integrated *Liriomyza* leafminers management in *P. vulgaris* production system. The endophytic fungi *H. lixii* F3ST1 and *B. bassiana* G1LU3 caused detrimental effects to LMF in the field by affecting larval development, resulting lower number of pupae, with no documented adverse effects on associated parasitoid populations. This resulted to good management of *Liriomyza* leafminer flies in the field leading to total avoidance in use of synthetic pesticides as a management strategy of LMF. Consequently, high yields were obtained from the endophytically-colonized *P. vulgaris* plants combined with *M. anisopliae* ICIPE 20 applied in autodissemination device traps. Results of the present study suggest that both fungal isolates (*H. lixii* F3ST1 and *B. bassiana* G1LU3) hold potential for pest management and could be considered for the control of *Liriomyza* leafminer flies. The two strategies are cost effective and can be embraced by both small and large scale farmers. However, there is the need to confirm these results on large-scale trials.

5.2 Conclusion

Fungal endophytes in combination with *M. anisopliae* ICIPE 20 applied in autodissemination device and parasitoids can contribute to integrated *Liriomyza* leafminers management in *P. vulgaris* production system. The endophytic fungi *H. lixii* F3ST1 and *B. bassiana* G1LU3 were found to cause detrimental effect to *Liriomyza* leafminers at the field by causing larval mortality and reducing number of pupae, with no adverse effects on the associated parasitoids populations. Consequently, high yields were obtained from the endophytically-colonized *P. vulgaris* plants combined with *M. anisopliae* ICIPE 20 applied in auto dissemination device traps.

5.3 Recommendations

From the above results, I can then recommend that:

1. Further studies are warranted on large-scale trials.
2. Further work should be done to establish colonization of *P. vulgaris* plants in the subsequent generations using the harvested seeds.
3. Further studies are warranted to assess *P. vulgaris* bean plants growth stimulation by the endophytic fungi at the field level.

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