Interrelationships between *Diglyphus isaea, Phaedrotoma scabriventris* and endophytic fungi in the control of *Liriomyza* leafminers

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Thesis submitted for the degree Doctor of Philosophy in Environmental Sciences at the Potchefstroom Campus of the North-West University

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September 2013

It all starts here

NORTH-WEST UNIVERSITY
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Interrelationships between *Diglyphus isaea*, *Phaedrotoma scarbiventris* and endophytic fungi in the control of *Liriomyza* leafminers

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Thesis submitted in fulfilment of the requirements for the award of the degree Doctor of Environmental Sciences at the North-West University (Potchefstroom Campus)

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April 2013

NORTH-WEST UNIVERSITY, SOUTH AFRICA

DEDICATION

To my beloved parents Komi Venyo Akutse and Aku Agnes Agbeke-Akutse for their constant sacrifices and supports

To my beloved wife Ama E. Genevieve Amenya-Akutse and our dear daughter Esther Sitsofe A. Akutse for their encouragements and love

To the Late Dr. A. Chabi-Olaye for conceptualizing this research topic and for his various advice, assistance and supports. This is a fruit of your dreams on this project of tackling *Liriomyza* leafminers

DECLARATION AND APPROVAL

Declaration by the candidate

I, **Komivi Senyo Akutse**, declare that this research project which I hereby submit for the degree of *Philosophiae Doctor* (Environmental Sciences and Development) at the North-West University is entirely my original work and has not been submitted for a degree in any other University.

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Approval by supervisors

The supervisors of this study give permission that the data generated during the study may be used for scientific publication by the student.

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ABSTRACT

Horticulture is a major foreign exchange earner in Kenya and provides employment to approximately 75% of the population. However, the growth of the horticultural industry is constrained by pests such as the leafminer flies, *Liriomyza sativae*, *L. trifolii* and *L. huidobrensis* (Diptera: Agromyzidae). These pests do not only cause damage to crops, but are also tagged as quarantine pests, resulting in export rejections, loss of export markets and consequently loss of revenue to smallholders. The management of leafminers worldwide has commonly relied on the use of chemical insecticides, but due to associated negative effects thereof, biological control using parasitoids and entomopathogenic fungi has been proposed as major components of integrated pest management (IPM) strategies. The indigenous ectoparasitoid *Diglyphus isaea* and exotic endoparasitoid *Phaedrotoma scabriventris* are the two key natural enemies being considered. A number of endophytic fungal isolates have been identified with potential for use as biological control agents of pests. Although there have been previous reports on toxicity of fungal endophytes to leafminers, no attempts have been made to exploit them for control of *Liriomyza* leafminers. The objectives of this study were to investigate the mechanisms by which fungal endophytes control *Liriomyza* spp., as well as the interactions between these endophytic fungi and the *L. huidobrensis*, endoparasitoid *Phaedrotoma scabriventris* and ectoparasitoid *Diglyphus isaea*. This study showed that under laboratory conditions, while used separately, parasitism rates of *L. huidobrensis* by *D. isaea* and *P. scabriventris* were 63.6 ± 7.7% and 30.4 ± 10.9% respectively and increased to 77.0 ± 5.3% when used simultaneously. In addition, both parasitoids induced leafminer mortality through larval-feeding and stinging. In order to identify and characterize endophytic fungi that could possibly be used for control of these pests, fungi were isolated from the aboveground parts of maize, sorghum, Napier grass, Coleopteran larvae and *Busseola fusca* pupae. Identified fungi were evaluated endophytically in two host plants species (*Phaseolus vulgaris* and *Vicia faba*) through seed inoculation. The fungal isolates that succeeded in colonizing the host plants were all pathogenic to *L. huidobrensis,* causing 100% mortality within 13.2 ± 0.7-15.0 ± 0.6 days. They were also able to reduce the longevity of the progeny, the number of pupae and adult emergence and survival. In addition, results also showed that endophytically-

inoculated and *L. huidobrensis*-infested *V. faba* plants had no adverse effects on parasitism rates and life history parameters of *P. scabriventris* and *D. isaea.* Bio-prospecting for fungal endophytes in *P. vulgaris* and *V. faba* seeds, followed by morphological and molecular identification revealed the presence of various species of fungal entomopathogens, including *Beauveria bassiana*, *Epacris microphylla*, *Phanerochaete chrysosporium* and *Metarhizium anisopliae*.

Key words: Biological control, entomopathogens, endophytes, horticulture, leafminers, *Liriomyza* sp., parasitoids, *Phaedrotoma scabriventris*, *Diglyphus isaea*

UITTREKSEL

Tuinbou is ‘n belangrike verdiener van buitelandse valuta in Kenia en verskaf werk aan ongeveer 75% van die bevolking. Ontwikkeling van die tuinbou-industrie word egter beperk deur insekplae soos die bladmynervlieë *Liriomyza sativae*, *L. trifolii* en *L. huidobrensis* (Diptera: Agromyzidae). Hierdie plae beskadig nie alleen gewasse nie maar is ook geïdentifiseer as kwarantynplae, wat gevolglik lei tot afkeur van uitvoerprodukte, verlies aan uitvoermarkte en gevolglike verlies aan inkomste vir kleinboere. Die bestuur van bladmyners wêreldwyd het grootliks berus op die gebruik van chemiese insekdoders maar as gevolg van die geassosieerde negatiewe effekte daarvan word biologiese beheer deur die gebruik van parasitoïede en entomopatogeniese fungi voorgestel as belangrike komponente van geïntegreerde plaagbestuursprogramme. Die inheemse ektoparasiet, *Diglyphus isaea* en uitheemse endoparasiet, *Phaedrotoma scabriventris* is die twee natuurlike vyandspesies wat hiervoor oorweeg word. ‘n Aantal endofitiese fungi-isolate met potensiaal vir gebruik as biologiese beheeragente is geïdentifiseer. Alhoewel daar voorheen toksisiteit van fungus-endofiete op bladmyners waargeneem is, is nog geen pogings aangewend om hierdie organismes vir die beheer van *Liriomyza*-bladmyners aan te wend nie. Die doelstellings van hierdie studie was om ondersoek in te stel na die volgende: die meganismes waardeur fungus-endofiete *Liriomyza* spp. beheer, die interaksies tussen hierdie endofiete en *L. huidobrensis*, die endoparasitoïed *Phaedrotoma scabriventris* en ektoparasitoïed *Diglyphus isaea.* Hierdie studie het getoon dat, onder laboratoriumomstandighede, indien parasitoidspesies individueel gebruik word teen *L. huidobrensis*, parasitisme deur *D. isaea* en *P. scabriventris* respektiewelik 63.6 ± 7.7% en 30.4 ± 10.9% was en dat dit toeneem tot 77.0 ± 5.3% indien die spesies saam voorkom. Verder is waargeneem dat beide spesies bladmynermortaliteit veroorsaak deur larwale-voeding en steekgedrag. In ‘n poging om endofitiese fungi te identifiseer en karakteriseer wat moontlik vir beheer van hierdie plae gebruik kan word, is fungi geïsoleer uit die bogrondse dele van sorghum, Napier gras, Coleoptera larwes en *Busseola fusca* papies. Geïdentifiseerde fungi is endofities geëvalueer in twee gasheerplantspesies (*Phaseolus vulgaris* en *Vicia faba*) deur saadinokulasie. Die

fungus-isolate wat die gasheerplante suksesvol koloniseer het was almal patogenies vir *L. huidobrensis,* en het gely tot 100% mortaliteit binne 13.2 ± 0.7-15.0 ± 0.6 dae. Hierdie fungi het ook die lewensduur van die nageslag verkort asook die aantal papies en volwassenes wat vorm en oorlewing verminder. Resultate het ook aangedui dat endofities-geinokuleerde en *L. huidobrensis*-geinfesteerde *V. faba* plante geen nadelige effek gehad het op parasitismevlakke en lewensparameters van *P. scabriventris* of *D. isaea* nie. Bioprospektering vir fungusendofiete in *P. vulgaris* en *V. faba* sade, gevolg deur morfologiese en molekulêre identifikasie het getoon verskeie spesies entomopatogeniese fungi, insluitend *Beauveria bassiana*, *Epacris microphylla*, *Phanerochaete chrysosporium* en *Metarhizium anisopliae* in sade voorkom.

Sleutelwoorde: Biologiese beheer, entomopatogene, endofiete, tuinbou, bladmyners, *Liriomyza* sp., parasitoïede, *Phaedrotoma scabriventris*, *Diglyphus isaea*

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CHAPTER 1: GENERAL INTRODUCTION

1.0 Introduction

The Agromyzidae leaf mining flies belong to a diverse dipteran family of exclusively phytophagous species which can be leaf and stem miners, seed parasites and gall inducers (Dempewolf, 2004). A number of species are of economic importance, especially those feeding on horticultural plants (Musundire *et al.*, 2010). The genus *Liriomyza* leaf mining flies is believed to be of neotropic origin with their distribution being restricted to the New World until the mid 1970s. From the warmer parts of the New World, some members of the genus have subsequently spread to Africa, Asia, Latin America and various oceanic islands (Murphy and LaSalle, 1999; EPPO, 2006). The most common invasive *Liriomyza* species, frequently reported from Africa are *L. sativae* (Blanchard), *L. trifolii* Burgess and *L. huidobrensis* Blanchard (Chabi-Olaye *et al.*, 2008). These three species, which are highly polyphagous, attack vegetable and ornamental plants in many parts of the world (Chaput, 2000). The serpentine leafminer, *L. trifolii,* was accidentally introduced into Kenya during 1976 through chrysanthemum (*Chrysanthemum* spp.; Asterales: Asteraceae) cuttings from Florida, USA, and has subsequently spread from the coastal areas to the highlands as well as to many African countries (Spencer, 1985). In Kenya, damage by leafminers has been recorded on various vegetable crops and ornamental plants belonging to the Compositae, Solanaceae, Cucurbitaceae, Malvaceae, Alliaceae, Passifloraceae and Caryophyllaceae families (Kabira, 1985; KEPHIS, 2005). Considerable leafminer damage has been reported from snowpea, *Pisum sativum* L. (Fabaceae), runner bean, *Phaseolus coccineus* L. (Fabaceae), French bean, *Phaseolus vulgaris* L. (Fabaceae), okra, *Abelmoschus esculentus* (Malvaceae), and cut flowers. Yield losses caused by *Liriomyza* leafminers can range between 20-100%, depending on crop, level of infestation and location (OEPP/EPPO, 1994).

Leafminers damage crops by puncturing the leaf surface to feed on exuding sap and ovipositing into the leaf tissue (Knodel-Montz *et al*., 1985). When the eggs hatch, larvae tunnel within the leaf tissue forming damaging and disfiguring mines. Leaf mines and punctures result in reduction of 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). In addition to their damage and losses, *Liriomyza* leafminers are also listed as quarantine pests in the EU Plant Health Directive 2000/29 (EU, 2000). These pests therefore prevent new market opportunities for Kenyan horticultural producers due to strict quarantine requirements by the overseas markets.

Several management strategies are used by both smallholder and large-scale producers. However, the main control strategy is the use of synthetic chemical insecticides such as carbamates, organophosphates and pyrethroids (Murphy and LaSalle, 1999). Indiscriminate and frequent use of these synthetic chemical insecticides for control of leafminers has resulted in negative impacts on natural enemies, environmental contamination, health risks, pesticide residues and development of resistance to insecticides (MacDonald, 1991; Weintraub and Horowitz, 1995; Murphy and LaSalle, 1999). These adverse effects have prompted the development of non-chemical methods such as biological control (parasitoids, entomopathogenic nematodes and entomopathogenic fungi) (Walters *et al*., 2000; Migiro *et al*., 2010), trapping by yellow sticky traps (Price *et al*., 1981) and resistant plant varieties (Hanna *et al.*, 1987).

Biological control using parasitoids and entomopathogenic fungi (EPF) is the strategy being developed at the International Centre of Insect Physiology and Ecology (*icipe*) for the management of these invasive leafminer species. The ectoparasitoid *Diglyphus isea* Walker (Hymenoptera: Eulophidae) which is used as a biological control agent against *Liriomyza* species in Europe, U.S.A. and some parts of Asia has also been reported in some parts of Africa including Kenya (Musundire, 2011) where large-scale mass-production programs have been initiated. Recently, the endoparasitoid *Phaedrotoma scabriventris* Nixon (Hymenoptera: Braconidae) was introduced from South America for classical biological control of the leafminer flies. The virulence of fungal isolates against

Liriomyza leafminer adults was reported in the laboratory by Migiro *et al*. (2010). Entomopathogenic fungi are generally applied in inundative approach in the crop (Lacey and Goettel, 1995). However, other strategies are currently being considered and include autodissemination (Vega *et al*., 2007) and endophytic colonization (Vega *et al*., 2009). The prospects of autodissemination for the control of *L. huidobrensis* were recently demonstrated in cage field experiments by Migiro *et al*. (2010). Fungal pathogens can endophytically colonize host plant and confer resistance against insect pests (Vega *et al*., 2009). Their potential for the control of leafminers was recently reported (J. Akello, N.K. Maniania, A. Chabi-Olaye and R. Sikora, unpublished data).

The objectives of this study were therefore to investigate the effects of the endophytically-inoculated host plants on *Liriomyza* spp., as well as the interactions between these endophytic fungi and the *Liriomyza huidobrensis*, endoparasitoid *Phaedrotoma scabriventris* and ectoparasitoid *Diglyphus isaea* in order to improve biological control of these pests.

1.1 Statement of the problem and justification

Horticulture presents enormous economic opportunities (income and food) for improving livelihoods in many countries in Africa. The production of horticulture crops is, however, severely constrained by infestation of *Liriomyza* spp. Yield losses are estimated to range between 20-100%, depending on crop, level of infestation and location. The most damaging species are *L. sativae, L. trifolii* and *L. huidobrensis*. The control of *Liriomyza* species is difficult because of their high degree of polyphagy and their resistance against several synthetic chemical pesticides used in the production systems (Chabi-Olaye *et al.*, 2008; OEPP/EPPO, 1994). Frequent use of synthetic chemical insecticides has increased the pest status due to elimination of natural enemies and the ability of *Liriomyza* leafminers to develop resistance against these insecticides. In addition, *Liriomyza* leafminers are considered as quarantine pests, resulting in export restrictions of *Liriomyza* leafminers-infested crops to important overseas markets. This has prompted the need to develop alternative management strategies that are effective and

environmentally-friendly. A strategy for inundative release of the most dominant indigenous parasitoid *D. isaea* in Kenya has been developed and implemented. *Phaedrotoma scabriventris* was recently introduced into Kenya as classical biocontrol agent of *Liriomyza* leafminers and has successfully established. Recent studies have also shown that fungal endophytes have potential for the control of leafminers (J. Akello, N.K. Maniania, A. Chabi-Olaye and R. Sikora, unpublished data). These components could be integrated together for effective management of *Liriomyza* leafminers. There is the need therefore to investigate host endophytes-plant-leafminer-parasitoid interactions as well as endoparasitoid-ectoparasitoid interactions. For instance, fungal endophytes by conferring resistance of host plant to insects may also interact with the insect host and natural enemies. The proposed project is part of *icipe*'s program that investigates new IPM strategies to address the world-wide expansion of *Liriomyza* leaf-mining flies and the associated huge losses in horticultural production in Kenya. The project focuses on strategies that support the self-regulation of agro-ecosystems and seeks sustainable solutions through the integration of biological control, based on the use of parasitoids, entomopathogens. Among the entomopathogens, the use of fungal endophytes is being considered.

1.2 Objectives

1.2.1 General objective

The general objectives of this study were therefore to investigate the mechanisms by which fungal endophytes control *Liriomyza* spp., as well as the interactions between these endophytic fungi and the *Liriomyza huidobrensis*, the endoparasitoid *Phaedrotoma scabriventris* and ectoparasitoid *Diglyphus isaea* in order to improve biological control of these pests.

1.2.2 Specific objectives

To attain this general objective, the following specific objectives are proposed:

1) Screen fungal pathogen isolates for their endophytic colonization of preferred *Liriomyza* spp. host plants.

2) Determine the effects of endophytic pathogens on *Liriomyza* spp.

3) Investigate the interaction between the endoparasitoid *P. scabriventris* and the ectoparasitoid *D. isaea* and the implication of such competition in biological control of *Liriomyza* spp.

4) Investigate the interactions between endophytically colonized host plant, *Liriomyza* spp. and the parasitoids, *Diglyphus isaea* and *Phaedrotoma scabriventris*.

1.3 Research hypotheses

1) The process of searching and colonization of the ectoparasitoid *D. isaea* and the endoparasitoid *P. scabriventris* leads to competition between the two parasitoids.

2) Endophytic fungi adversely affect the developmental stages of *Liriomyza* spp.

3) Interactions between endophytic fungi and parasitoids may affect biological control of *Liriomyza* spp.

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CHAPTER 2: LITERATURE REVIEW

2.0 The genus *Liriomyza*

The genus *Liriomyza* (leafminer flies) is believed to be of neotropic origin and was restricted to the New World until the mid 1970s. Since then, several species of the genus have been spreading to other regions and are presently reported in several countries of Africa, Asia and Latin America. They belong to the Agromyzidae family which consists of about 2,750 species (Tschirnhaus, 2000). The Agromyzidae are exclusively phytophagous and their larvae which are internal feeders can be leaf miners, stem miners, seed parasites and gall inducers (Dempewolf, 2007). Leaf mining is generally the most widespread feeding behaviour shared by approximately 75% of the species (Spencer, 1973). A number of species are of economic importance, especially those feeding on horticultural plants. The genus *Liriomyza* comprises about 330 species (Liu *et al.*, 2009) of which only 6% are economically important (Liu *et al*., 2009). These include *L. trifolii, L. sativae, L. bryoniae, L. strigata*, and *L. huidobrensis*. In Kenya, *L. huidobrensis, L. sativae* and *L. trifolii* are the three most important species in the horticultural sector, with *L. huidobrensis* being the most aggressive / injurious one (Figure 2.1).

Figure 2.1: The three main species of *Liriomyza* identified in Kenya: *Liriomyza huidobrensis* (a), *Liriomyza sativae* (b) and *Liriomyza* t*rifolii* (c).

2.1 The biology of the genus *Liriomyza*

Morphological identification of the three leafminer species (Figure 2.1) is based on the distiphallic structure, a terminal part of the aedaegus (Chaput, 2000). *Liriomyza huidobrensis* are distinguished from other *Liriomyza* species particularly *L. sativae* and *L. trifolii* by larger body size, overall dark colour (Figure 2.1), larger discal cell, relatively short distal section of vein M3+4, darkened femora (yellow in *sativae* and *trifolii*) and the male genitalia (OEPP/EPPO, 2005; Collins, 2009).

Mating, which is mainly observed during morning hours, takes place from 24 hours after emergence and a single mating is sufficient to fertilize all eggs (Parrella, 1987). Female flies puncture leaves of the host plants causing wounds which serve as sites for feeding or oviposition. Feeding punctures cause the destruction of a large number of cells, and are visible to the naked eye as white speckles measuring between 0.13 and 0.15 mm in diameter. Oviposition punctures are smaller (0.05 mm) and are more uniformly round than feeding punctures (EPPO/CABI, 2006). About 15% of punctures made by *L. sativae* and *L. trifolii* contain viable eggs (Parrella *et al*., 1981). Males are unable to puncture leaves but have been observed to feed at punctures produced by females. The pre-oviposition period, which may extend to five days, is determined by temperature, relative humidity and availability of food (Parrella, 1987). Feeding and oviposition occur most commonly during the morning hours and the frequency of activities is positively correlated to temperature (Fagoonee and Toory, 1984). Mean egg production per female ranges from less than 100 to more than 600, depending on environmental conditions, hosts and leafminer species (Parrella, 1987). According to Chaput (2000), optimal temperatures for egg laying of the three mentioned species range between 21 and 32 °C and egg laying is reduced at temperatures below 10 °C. In younger females, eggs are laid at a rate of 30 to 40 per day, with numbers decreasing as flies age (Mau and Kessing, 2000). The size of *Liriomyza* spp. eggs is 0.2-0.3 mm x 0.10-0.15 mm. Eggs have an off-white colour and are slightly translucent. Eggs are inserted just below the leaf surface (Figure 2.2) and hatch 4 – 7 days after oviposition depending on temperature. There are three larval stages, each of which is completed in

2 to 3 days. Pupal development is completed in 5 to 12 days, whereupon adults emerge from pupae, principally in the early morning hours (Mau and Kessing, 2000).

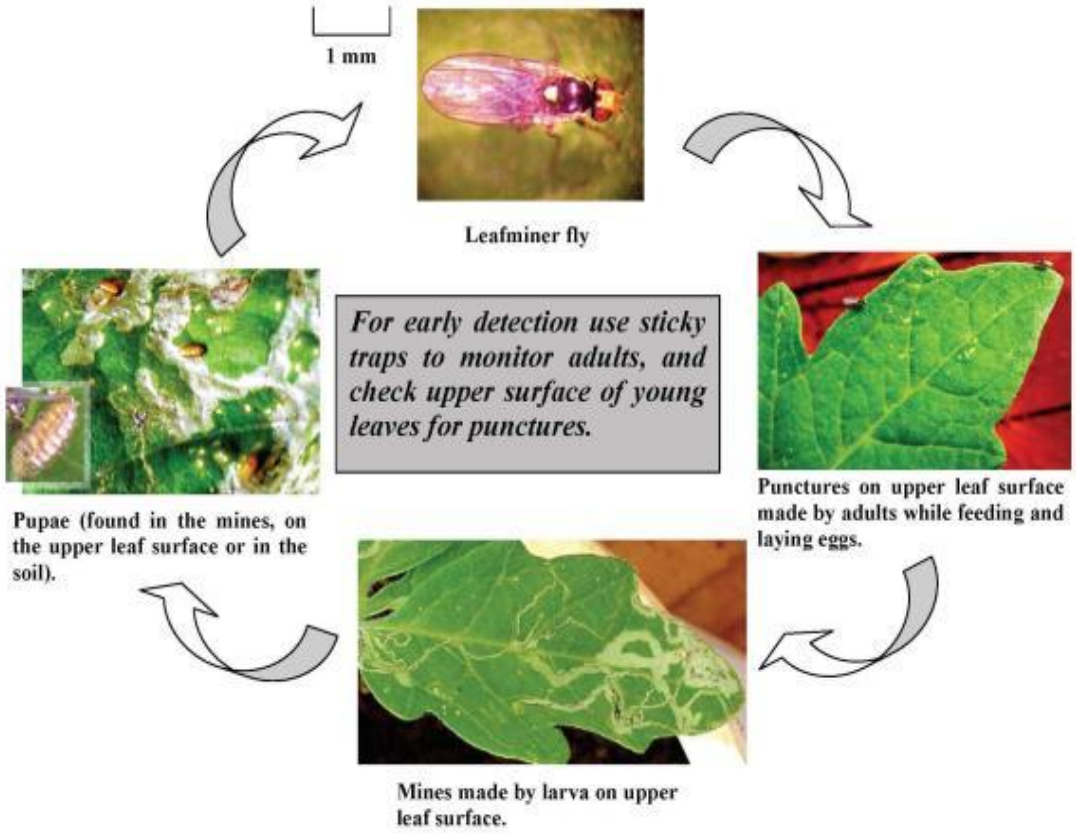

Figure 2.2: *Liriomyza* leafminer life cycle. © A. M. Varela*, icipe* (Varela *et al*., 2003). http://www_infonet-biovision_org - Leafmining flies (leafminers).mht

Duration of the life cycle varies with host and temperature. The average life cycle is approximately 21 days in warm conditions, but can be as short as 15 days. Populations can therefore increase rapidly (Varela *et al.,* 2003; CABI, 2004). Under greenhouse conditions at 27°C, the egg stage of *L. huidobrensis* lasts 3 days, larval stages 3 to 5 days and the pupal stage 9 days (Parrella and Bethke, 1984). Development time required by *L. sativae* egg and larval stages is about 7 to 9 days at 25-30°C while pupal development takes about 9 days at the same temperatures under laboratory conditions (Capinera, 2007). Under laboratory conditions at 25°C, eggs of *L. trifolii* requires about

3 days for development, while the larval stages requires about 5 days and pupal stage about 9 days (Minkenberg, 1989).

2.2 Geographical distribution of the pest in Africa

Liriomyza flies are serious pests of vegetables and ornamental plants worldwide. Adult flies are capable of limited flight. Long-distance dispersal is facilitated by movement of planting material of host species. Cut flowers can also present a danger as a means of dispersal. *Liriomyza* species have been reported in several African countries, including Kenya, Mauritius, Reunion, Senegal, South Africa, Uganda and Tanzania (Figure 2.3). While *L. trifolii* was first introduced to Kenya in the late 1970s through chrysanthemum cuttings from Florida, USA (Spencer, 1985), *L. sativae* was only recorded recently (Chabi-Olaye *et al*., 2008).

Figure 2.3: Geographical distribution of *Liriomyza* leaf mining flies in Africa (countries indicated in red are where *Liriomyza* species have been reported from). http://www_infonet-biovision_org - Leafmining flies (leafminers).mht

2.3 Economic importance of *Liriomyza* leafminers

Liriomyza sativae has been reported to damage a wide range of vegetables including tomatoes, potatoes and *Cucurbit* spp. It also transmits a number of plant viruses, including celery mosaic potyvirus (Zitter *et al*., 1980). *Liriomyza trifolii* is the major pest of chrysanthemums, celery and onions in North America (Foster and Sanchez, 1988; EPPO/CABI, 2006) with losses for celery estimated at US$ 9 million in 1980 (Spencer, 1982). *Liriomyza trifolii* is also known to vector plant viruses (Zitter *et al*., 1980). On the other hand, *L. huidobrensis* is a key pest of potato, glasshouse ornamentals and vegetable crops (OEPP/EPPO, 1994) and is considered a more serious pest than *L. trifolii* in Israel (Weintraub and Horowitz, 1995). *Liriomyza huidobrensis* has been reported as serious pest of horticulture especially on ornamentals and passion fruits (KEPHIS, 2005).

Damage by leafminers is caused by larvae mining into leaves and petioles. The photosynthetic ability of the plants is often greatly reduced as the chlorophyll-containing cells are destroyed. Severely infested leaves may fall, exposing plant stems to wind and flower buds and developing fruit to scald (Musgrave *et al*., 1975). The presence of unsightly larval mines and adult punctures in the leaf palisade of ornamental plants can further reduce crop value (Musgrave *et al*., 1975). In young plants and seedlings, mining may cause considerable delay in plant development leading to plant loss.

2.4 Control strategies for leafminer flies

2.4.1 Chemical control

The most widely reported reason for the first leafminer outbreaks in their adventive ranges was the indiscriminate use of insecticides which adversely affected their natural enemies (Murphy and LaSalle, 1999). Many horticultural growers in Kenya have been using avermectins (abamectin), triazines (cyromazine), carbamates, organophosphates and pyrethroids to control leafminers (Kabira, 1985). These synthetic chemical insecticides are however no longer very effective due to resistance development (Musundire *et al.*, 2010). Kotzee and Dennill (1996) also reported resistance of *L. trifolii*

to cyromazine and triazine in South Africa. Resistance of *Liriomyza* leafminers to most carbamate, organophosphate, and pyrethroid insecticides has also been reported in the United Kingdom (MacDonald, 1991).

2.4.2 Biological control

Parasitoids

Parasitoids recorded from *L. huidobrensis*, *L. sativae* and *L. trifolii* from around the world are diverse and include species in the hymenopteran families Eulophidae, Pteromalidae, Eucoilidae and Braconidae. A koinobiont parasitoid, *Phaedrotoma scabriventris* Nixon (Braconidae: Opiinae), attacking leafmining larvae, has been mentioned as the most important parasitoid of these leafminers, causing 20 - 51% mortality in Argentina, Brazil and Peru (Valladares *et al.*, 1999; Kroschel, 2008). In Argentina, Colombia, Mexico and Peru, *Chrysocharis caribea* Boucek (Hymenoptera: Eulophidae) is an extremely important mortality factor in agromyzid leafminer populations with an average of 30 – 55% parasitism (Kroschel, 2008; Valladares *et al*., 2001). *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae), and *Halticoptera arduine* (Walker) (Hymenoptera: Pteromalidae) have been reported to cause 35 – 73% mortality to *Liriomyza* leafminers in Chile, Peru and Argentina (Murphy and LaSalle, 1999; Kroschel, 2008). *Dacnusa sibirica* Telenga (Leuprecht, 1993), *Opius pallipes* Wesmael and *Diglyphus isaea* (Van der Linden, 2004; Benuzzi and Raboni, 1992) are under consideration for use as natural enemies of the pest in European glasshouses. Amongst these parasitoids, *D. isaea* has been shown to be effective at higher temperatures (Minkenberg, 1989) and thus, could be effectively used in controlling leafminers in tropical environments. In Africa, large-scale mass-production programmes of *D. isaea* have been developed to support biological control of leafminer efforts in Kenya and South Africa through augmentative biological control approaches (A. L. Owuor, Dudutech Pvt. Ltd.- Kenya, pers. comm.; Musundire *et al*., 2010). *Diglyphus begini* (Ashmead) is also used in United States for augmentative biological control of leafminers (Sher *et al*., 2000).

Entomopathogenic fungi and nematodes

There are only few reports on the potential of entomopathogenic fungi (EPF) and nematodes as biocontrol agents of leafminers (Harris *et al*., 1990; Walters *et al*., 2000; Migiro *et al*., 2010; Wekesa *et al*., 2010). For example, foliar applications of the entomopathogenic nematode *Steinernema carpocapsae* (Weiser) significantly reduced adult development rate of *L. trifolii* (Harris *et al*., 1990). Head *et al*. (2000) reported the potential of using an entomopathogenic nematode *Steinernema feltiae* in combination with chemical pesticides in an integrated pest management (IPM) program. Application of *Isaria fumosorosea* Wise was reported to reduce leaf mines by *L. trifolii* on gerbera and sunflower as compared to pesticide treatment (Wekesa *et al*., 2010).

IPM approaches based on conservation of existing natural enemies and introductions of additional species may provide viable alternatives to the application of insecticides (Kang *et al*., 2009).

Cultural practices and plant resistance to Liriomyza leafminer damage

Crops may vary in their susceptibility to leaf miner damage. This has been noted, for example, in cultivars of tomato, cucumber, cantaloupe and beans (Hanna *et al*., 1987). However, the resistance tends to be moderate and not adequate for reliable protection. Nitrogen levels in leaves and reflective mulches have been reported to influence leafminer populations (reduction of their population density), but responses have not been consistent (Chalfant *et al*., 1977; Hanna *et al*., 1987). Placement of row covers over cantaloupe has been reported to prevent damage by leafminer (Orozco-Santos *et al*., 1995). Hand- picking, destruction of mined leaves and other plant material after harvest has been found to significantly suppress leafminer damage (Varela *et al*., 2003). Varela *et al*. (2003) also reported that ploughing and flooding of the soil, followed by hoeing could kill or expose much of the buried pupae, which are then killed by solarization or exposure to natural enemies.

Monitoring of Liriomyza leafminer flies

Several methods of leafminer population assessment have been studied and regular monitoring is needed to maintain currency in addressing problems caused by the *Liriomyza* species complex. Collecting puparia in trays placed beneath plants was recommended by Johnson *et al*. (1980) as a labor-saving technique. Leaf miners can also be monitored by foliage examination for the presence of mines and larvae. Sticky traps should be placed in and around the borders of the fields at about 10 cm above the foliage to monitor leafminers population (Braun and Merle, 1997). The value of pupal counts during monitoring for prediction of adult numbers two weeks later is also recommended (Zehnder and Trumble, 1984). Sequential sampling plans were developed by Zehnder and Trumble (1985) and visual rating systems to assess the total number of leaf miners on tomato have been developed in the USA (Varela *et al*., 2003; Koppert, 2003).

2.4.3 Post-harvest treatment

To avoid the introduction of *Liriomyza* species from countries where they occur, propagating materials must be inspected at least every month for three months prior to export, and produce have to undergo post-harvest treatments (EPPO/CABI, 1996; OEPP/EPPO, 1990). A phytosanitary certificate is required for cut flowers and for vegetables with leaves.

Cold-treatment have also been deployed as control measure. All stages of *Liriomyza* larvae are killed after 1-2 weeks at 0°C (Webb and Smith, 1970). Newly laid eggs are, however, the most resistant stage. *Liriomyza trifolii* eggs in chrysanthemums can survive for up to three weeks in cold storage at 0°C and for at least 10 days at 1.7°C (Webb and Smith, 1970). Eggs incubated for 36-48 hours were killed after one week under the same conditions. It was therefore recommended by these authors that cuttings of infested ornamental plants be maintained under normal glasshouse conditions for 3-4 days after lifting, to allow eggs to hatch. Subsequent storage of the plants at 0°C for 1-2 weeks should then kill off the larvae (Webb and Smith, 1970).

Gamma irradiation of eggs and first larval stages at doses of 40-50 Gy provided effective control, but lower doses were ineffective (Yathom *et al*., 1991).

2.5 The biology of leaf miner parasitoids

2.5.1 The ectoparasitoid *Diglyphus isaea*

The adult parasitic wasp *D. isaea* is small (2 mm long) and black with a metallic green sheen (Bouček, 1988; Anonymous, 2007) (Figure 2.4).

Figure 2.4: Female *Diglyphus isaea* searching *Liriomyza huidobrensis* larvae for oviposition.

Diglyphus isaea perform better in warmer conditions. Prior to oviposition, females paralyze leafminer larvae by introducing a toxin. The toxic effect is immediate and larvae stop feeding. These small wasps also feed on the host larvae (host-feeding), as do many wasps. After feeding the female lays 1-5 eggs next to the paralyzed larva. A female lays an average of 50 - 60 eggs during her lifespan. The parasitoid larvae hatch within 2 days and while feeding externally on the leafminer larva (ectoparasitism)

(Haghani *et al*., 2010), they pass through three instars during the following six days (Anonymous, 2007). After consuming the leafminer larva, the mature parasitoid larvae turn into turquoise coloured pupae within 9 days. Before pupa formation, the larva places its faeces (meconia) in a typical symmetrical pattern on both sides of its body. The parasitoid larva then constructs pillars of faecal matter around the remains of the much deteriorated pest larva. These are thought to protect the beneficial larvae inside the mines of desiccating leaves while they undergo pupation. Pupae turn black before adults emerge 6 to 9 days later through a hole chewed in the upper surface of the leaf. At 20 °C female *D. isaea* larvae develop from eggs to pupae in 9 days (Minkenberg, 1989). The pupal stage at this temperature lasts 8 days. At 15 °C development time from egg to adult is 26-27 days, whilst it is shortened to 10-11 days at 25 °C. The development time of the parasitoid is shorter than that of most leafminer species (Bazzocchi *et al*., 2003). The adult parasitoid can also feed on the body fluids of leafminer larvae to obtain protein - an essential ingredient of its diet - to maintain egg production (Anonymous, 2007). The life-span of these parasitoids is approximately two weeks in their immature stages and three weeks as adults. The conditions for optimum performance are between 15 - 35 °C with a relative humidity of 80%. These are however, optimum conditions and not necessarily a prerequisite for successful completion of the life cycle. However, significantly cooler or warmer temperatures and humidity fluctuations may hamper reproduction and development (Haghani *et al*., 2010).

2.5.2 The endoparasitoid *Phaedrotoma scabriventris*

Phaedrotoma scabriventris Nixon (Hymenoptera: Braconidae) is a koinobiont parasitoid attacking leafmining larvae (Figure 2.5).

Figure 2.5: (a) Adult female of *Phaedrotoma scabriventris* with some morphological visible features and (b) two adults searching actively for *Liriomyza huidobrensis* larvae to parasitize

After completion of its cycle this parasitoid emerges from the puparia. This species is considered as the most important parasitoid of *Liriomyza* spp. and as a potential agent for its population regulation (Valladares *et al*., 1999). *Phaedrotoma scabriventris* developmental time (from eggs to adult) ranged between 12 days at 30ºC to 31.9 days at 15ºC. The lowest progenies develop at 10°C (36.2 progenies per female) and the highest at 15°C (151.2 progenies per female) (Mujica *et al*., 2009). At temperatures above 15° C progeny development is reported to decrease gradually. At 20°C up to 123 progenies per female can be produced (Mujica *et al*., 2009). The sex ratio is highly affected by temperature with female progeny reported to increase with increasing temperature. A female: male sex ratio of 0.66:1 at 10ºC and 1.31:1 at 30ºC have been reported with a balanced sex ratio of 1:1 recorded at 20ºC (Mujica *et al*., 2009).

2.6 Endophytes and their classification

Endophytes are heterotrophic microorganisms that live inside plants primarily for nutrition, protection and reproduction. Some of them are beneficial and some may be

pathogenic to crops (Carroll, 1988; Azevedo *et al*., 2000; Backman and Sikora, 2008). Fungal endophytes have been detected in hundreds of plants, including many important agricultural commodities such as wheat (Larran *et al*., 2002a), bananas (Pocasangre *et al*., 2000; Cao *et al*., 2002), soybeans (Larran *et al*., 2002b), and tomatoes (Larran *et al*., 2001). Some endophytes belong to genera that include fungal entomopathogens such as *Beauveria* (Ascomycota: Hypocreales). *Beauveria bassiana* (Balsamo) Vuillemin has been reported as an endophyte in maize (Cherry *et al*., 2004; Arnold and Lewis, 2005) and several other crops (Jones, 1994; Leckie, 2002; Ownley *et al*., 2004; Akello *et al*., 2007; Posada *et al*., 2007;).

Endophytes have been classified based on some criteria such as infested plant parts and host plants, taxonomy and structure, genetics, nutrition, reproduction, transmission, pathology and toxicology (Johnson *et al*., 1983; Schardl *et al*., 1991; Murray *et al*., 1992; Wilson, 1993; Brem and Leuchtmann, 2001; Araújo *et al*., 2002; Saikkonen *et al*., 2002; Narisawa *et al*., 2003; Karandashov *et al*., 2004). Understanding such bases would help to clarify the endophyte concept and assist in the utilization of such microbes in pest management.

Fungi such as certain *Fusarium* spp. and *Glomus* spp. that infect roots are 'root endophytes' (Wilberforce *at al*., 2003; Karandashov *et al*., 2004). These include 'root-invading' microbes that enter into plant tissues from the rhizosphere (Skipp and Christensen, 1989), as opposed to those that invade stems and leaves (foliar endophytes) (Wilson, 1993)

Endophytic organisms may form characteristic structures through their own cells or in tissues of host plants. Such features have been used to classify endophytes. The bacterial endophytes are prokaryotic as they lack nuclear membranes (Kamoun *et al*., 1998; Vellai and Vida, 1999), as opposed to eukaryotic endophytes that have nuclear membranes (Kupper *et al*., 2009). Most fungal endophytes, such as *F. oxysporum* V5w2 (Paparu *et al*., 2009a, b), are composed of threadlike structures known as hyphae that form mycelia masses, which earn them the name "mycelial endophytes" (Narisawa *et al*., 2003).

Molecular phylogenetic classification of endophytes involves the analysis of nucleic acids and proteins in studying the evolutionary relationships of endophytes. For example, through restriction fragment length polymorphism (RFLP) loci analysis, 72 RFLP haplotyes of *F. oxysporum* f. sp. *cubense* infecting banana have been identified (Koenig *et al*., 1997). A molecular phylogenetic relationship of *Epichloë typhina* and other clavicipitaceous endophytes, which also shows that endophytes may coevolve with their hosts, was reported by Schardl *et al*. (1991).

Endophytes which have been manipulated through genetic engineering are classified as "genetically modified endophytes" as opposed to the "wild-types" that retain their natural genomes (Murray *et al*., 1992; Gullino and Migheli, 1999). For example, *F. oxysporum* V5w2 a non-pathogenic endophyte was transformed with the green (GFP) and red fluorescent protein (DsRed) genes that facilitated its observation in banana root xylem since the wild-type could not be observed (Paparu *et al*., 2009a).

All endophytes are heterotrophs (organotrophs), since they acquire carbon in the form of organic compounds, unlike green plants that utilize CO_2 (Pace, 1997). They are also sub-classified as chemo-organotrophs since they utilize organic substances for energy, which contrasts their host plants that use light energy in photosynthesis (Johnson *et al*., 1983; Broda and Peschek, 1984). Depending on whether they gain nourishment from dead or living materials, endophytes can be classified as saprophytes, necrotrophs or biotrophs (Varma *et al*., 1999).

Based on the mode of reproduction, fungi as well as endophytes can be grouped as asexual or sexual (Brem and Leuchtmann, 2001). For example, the *Epichloë* endophytes have been divided into the genus *Epichloë* that reproduces sexually and the genus *Neotyphodium* (formally *Acremonium*) that only reproduces asexually (Moon *et al*., 1999; Leuchtmann *et al*., 2000; Schardl and Craven, 2003).

Endophytes are also classified based on their mode of transmission in host populations. Vertically-transmitted endophytes are passed directly from host plants to their offspring (Saikkonen *et al*., 2002). Those vertically transmitted via the host seeds are referred to as seed-transmitted endophytes (Dongyi and Kelemu, 2004), for example, *F. oxysporum* f. sp. *vasinfectum* race 4 in cotton (Bennett *et al*., 2008). On the other hand, horizontally-transmitted endophytes are contagiously transferred between different individuals in a population (Saikkonen *et al*., 2002). Such endophytes may be multiplied through vegetative propagules (Thomas *et al*., 2008), or transmitted via spores hence spore-transmitted endophytes (Faeth and Fagan, 2002).

The terms “symptomatic” and “asymptomatic (symptomless)” classify endophytes depending on whether the host plants express infection symptoms (Pinto *et al*., 2000; Araújo *et al*., 2002). For example, a wide range of endophytes especially *Fusarium* spp. were obtained from asymptomatic cord roots of banana cultivar Pisang Awak (*Musa* ABB) (Niere, 2001; Sikora *et al*., 2008). Symptomatic endophytes may sometimes be qualified as asymptomatic when the host plant is resistant (Araújo *et al*., 2002).

2.6.1 Endophytic fungi used for control of *Liriomyza* leafminers

It has recently been discovered that many entomopathogenic fungi play additional roles in nature. These fungi play roles such as endophytes, antagonists of plant pathogens, associated with the rhizosphere, and possibly even as plant growth promoting agents (Vega *et al*., 2009). Toxic activities of some fungal endophytes on leafminers have been reported (Faeth and Hammon, 1996; Gaylord *et al*., 1996; Preszler and Gaylord, 1996), but they are yet to be exploited for use in large-scale leafminer control.

Endophytes infect different plant organs and tissues, including roots, stems, branches, leaves, flowers, and fruits without causing symptoms, and they are garnering increased attention because they are ubiquitous and have immense diversity and varied roles (Saikkonen *et al*., 2006; Arnold and Lutzoni, 2007). Some fungal endophytes protect host plants against pathogens and herbivores (Arnold and Lewis 2005; Rudgers *et al*.,

2007), and many fungi traditionally known as insect pathogens have been isolated as endophytes, including species of *Acremonium, Beauveria, Cladosporium, Clonostachys* and *Isaria* (Vega, 2008; Vega *et al*., 2009).

2.7 Host, host plant, parasitoids and fungal endophyte interactions

Interactions between endophytic fungi, host plants, herbivores, and natural enemies were reviewed by Faeth and Bultman (2002). The interactions between herbivores, host plants and their natural enemies are best understood when considered within a tritrophic context (Kang *et al*., 2009; Zhao and Kang, 2002a, b). Host plant diversity, the volatiles that are released in response to feeding damage and leafminer-larval size are important factors in host finding and host selection by parasitoids (Takken and Dicke, 2006; Wei *et al*., 2007). The chemical compounds produced by plants which act as cues in long and short distance range (Dicke and Minkenberg, 1991) can be classified according to their effect on the host-location behaviour of insects. These categories are attractants, repellents, feeding and oviposition stimulants, and deterrents (Bernays and Chapman, 1994; Kang *et al*., 2009).

Jia-Ning *et al*. (2006) reported six different volatiles from *Liriomyza* infested bean plants (*Phaseolus vulgaris*). In addition, Kang *et al.* (2009) reported that (*Z*)-3-hexen-1-ol was the most important chemical emitted by plants that attract leafminer parasitoids, and that several other chemical volatiles play important roles in distinguishing host and non-host plants of *Liriomyza* species.

Plant quality and plant chemistry can be altered by the presence of microorganisms such as fungal endosymbionts (Clay, 1990). These endophytes produce different invertebrate- and vertebrate-toxic alkaloids. Several studies have shown increased resistance against herbivores of plants harbouring endophytes and associated negative effects on natural enemies (Meister *et al.*, 2006). However, not all herbivorous insects react the same way in the presence of endophytes (Faeth and Bultman, 2002; Saikkonen *et al.*, 2006; Härri, 2009). If endophyte-tolerant herbivores can use

mycotoxins as a type of acquired defense against their natural enemies, they could benefit from the presence of fungal endosymbionts. Leafminer feeding on endophyte-infected plants may represent lower quality hosts for parasitoids and parasitoid females may decide to place unfertilised, male eggs into such miner larvae if she can perceive the presence of the endosymbiont in the plants (Charnov *et al.*, 1981).

Several roles have been ascribed to fungal endophytes, including providing protection of plants against herbivorous insects (Breen, 1994; Clement *et al*., 1994), plant parasitic nematodes (West *et al*., 1988; Elmi *et al*., 2000), and plant pathogens (Dingle and McGee, 2003; Wicklow *et al*., 2005). Most reports on herbivore-endophyte interactions concentrated on turf and agronomic grasses. These fungi systemically infect mostly grasses in the Poaceae, Juncaceae, and Cyperaceae (Clay, 1990; Breen, 1994).
Some fungal endophytes protect host plants against plant pathogens (Ownley *et al.,* 2010) and herbivores, including insects (Arnold *et al.,* 2003; Arnold and Lewis, 2005; Schulz and Boyle, 2005; Rudgers *et al.,* 2007; Vega, 2008; Vega *et al.,* 2008). The negative effects of endophytic clavicipitalean fungi on insect herbivores have been generally ascribed to the production of fungal metabolites (Funk *et al*., 1983; Bush *et al*., 1997; Clay, 1988; Clay and Schardl, 2002). For example, exposure of two aphid species, *Rhopalosiphum padi* and *Metopopophium dirhodum* (Hemiptera: Aphididae) and wheat stem sawfly, *Mayetiola destructor* (Diptera: Chloropidae) to wild barley infected with *Neotyphodium coenophialum* (Hypocreales: Clavicipitaceae) reduced their survival (Clement *et al.,* 1994, 2005). Wheat leaves colonized by either *B. bassiana* or *Aspergillus parasiticus* (Eurotiales: Trichocomaceae) reduced the growth rate of *Chortoicetes terminifera* (Orthoptera: Acrididae) nymphs (Gurulingappa *et al.,* 2010). Endophytic *B. bassiana* in banana significantly reduced larval survivorship of banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), resulting in 42–87% reduction in plant damage (Akello *et al.,* 2008b). Reduction in feeding and reproduction by *Aphis gossypii* (Hemiptera: Aphididae) has also been reported on cotton endophytically colonized by either *B. bassiana* or *Lecanicillium lecanii* (Hypocreales: Clavicipitaceae) (Gurulingappa *et al.,* 2010). Another possible role for fungal endophytes could include plant growth promotion as well as impact on tritrophic

interaction (Akello *et al.,* 2007; Harish *et al.,* 2008; Vega *et al.,* 2008; Paparu *et al.,* 2009b). Fungal entomopathogens that become established as endophytes can therefore play an important role in the regulation of insect populations.

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CHAPTER 3: ENDOPHYTIC COLONIZATION OF *VICIA FABA* AND *PHASEOLUS VULGARIS* (FABACEAE) BY FUNGAL PATHOGENS AND THEIR EFFECTS ON THE LIFE-HISTORY PARAMETERS OF *LIRIOMYZA HUIDOBRENSIS* (DIPTERA: AGROMYZIDAE)

Abstract

Ten fungal isolates belonging to the genera *Beauveria* (3), *Hypocrea* (1), *Gibberella* (1), *Metarhizium* (2), *Trichoderma* (1) and *Fusarium* (2) were evaluated in the laboratory to determine whether they could become endophytic in two pea leafminer (*Liriomyza huidobrensis*) host plants (*Vicia faba* and *Phaseolus vulgaris*) and to assess their possible negative effects on leafminers. *Beauveria* (ICIPE279), *Hypocrea*, *Gibberella*, *Fusarium* and *Trichoderma* isolates colonized roots, stems and leaves of both host plant species. *Beauveria* isolates G1LU3 and S4SU1 colonized roots, stems and leaves of *P. vulgaris* but only the root and stem of *V. faba*. Isolates of *Metarhizium* failed to colonize the two host plants. The effects of endophytically-colonized fungal isolates on mortality, oviposition, emergence and longevity of *L. huidobrensis* were investigated after endophytic colonization of *V. faba* plants. All the fungal isolates that succeeded in colonizing the host plant were pathogenic to *L. huidobrensis,* causing 100% mortality within 13.2 ± 0.7-15.0 ± 0.6 days. However, *Hypocrea* outperformed the other isolates ($p < 0.0001$) in reducing longevity of the progeny (11.2 ± 1.0 vs. 17.8 ± 1.4 days in the control), the number of pupae (80.0 ± 6.7 vs. 387 0 ± 21.7 pupae in the control), and adult longevity (3.8 ± 1.0 vs. 9.9 ± 1.8 days in the control). Adult emergence was significantly reduced ($p < 0.0001$) in *Hypocrea* (21.4%) and *Beauveria* (38.0%) treatments as compared to the control (82.9%).

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3.0 Introduction

Liriomyza spp. (Diptera: Agromyzidae) leafminers are exotic pests of horticultural crops in Africa and have invaded large parts of the continent from the New World (Murphy and LaSalle 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). Yield losses of 20-100% have been reported, depending on crop and location (EPPO, 2006). The currently adopted management strategy for their control is largely based on use of synthetic chemical insecticides. In many cases pesticides are not effective due to the development of resistance, elimination of natural enemies and further pose health risks due to pesticide residues. Biological control through the use of parasitoids and fungal entomopathogens is being considered as part of an integrated strategy for leafminer management in Kenya (Migiro *et al.,* 2010). Fungal entomopathogens are generally applied in an inundative approach in the crop (Lacey and Goettel, 1995). The high cost of fungus production is a limiting factor for the adoption of the technology by growers, in addition to short survival of the inoculum in the environment. Another biocontrol strategy 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). It is now established that many of the fungal entomopathogens play additional roles such as endophytes in nature (Vega *et al.,* 2009). Fungal endophytes are heterotrophic microorganisms that live inside plants primarily for nutrition, protection and reproduction (Carroll 1988; Azevedo *et al.,* 2000; Backman and Sikora, 2008). They have been isolated from many crops including wheat (Larran *et al.,* 2002a), bananas (Pocasangre *et al.,* 2000; Cao *et al.,* 2002, 2005), soybeans (Larran *et al.,* 2002b), coffee (Vega *et al.,* 2008), and tomatoes (Larran *et al.,* 2001). Among the fungal entomopathogens, *Beauveria bassiana* (Balsamo) Vuillemin has been reported as an endophyte in maize (Bing and Lewis, 1991, 1992ab; Lomer *et*

al., 1997; Cherry *et al.,* 2004; Orole and Adejumo, 2009), banana (Akello *et al.,* 2008a), coffee (Vega *et al.,* 2008), tomato (Leckie, 2002; Ownley *et al.,* 2004) and cotton (Ownley *et al.,* 2004). *Metarhizium anisopliae* (Metchnikoff) Sorokin has been recorded as an endophyte on maize, Napier grass and beans (Akello, 2012).

Some fungal endophytes protect host plants against plant pathogens (Ownley *et al.,* 2010) and herbivores, including insects (Arnold *et al.,* 2003; Arnold and Lewis, 2005; Schulz and Boyle, 2005; Rudgers *et al.,* 2007; Vega, 2008; Vega *et al.,* 2008). For example, exposure of two aphid species, *Rhopalosiphum padi* and *Metopopophium dirhodum* (Hemiptera: Aphididae) and wheat stem sawfly, *Mayetiola destructor* (Diptera: Chloropidae) to wild barley infected with *Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, C.W. Bacon & Hanlin reduced their survival (Clement *et al.,* 1994, 2005). Wheat leaves colonized by either *B. bassiana* or *Aspergillus parasiticus* Speare (Eurotiales: Trichocomaceae) reduced the growth rate of *Chortoicetes terminifera* (Orthoptera: Acrididae) nymphs (Gurulingappa *et al.,* 2010). Endophytic *B. bassiana* in banana significantly reduced larval survival of banana weevil, *Cosmopolites sordidus* (Coleoptera: Curculionidae), resulting in 42–87% reduction in plant damage (Akello *et al.,* 2008b). Reduction in feeding and reproduction by *Aphis gossypii* (Hemiptera: Aphididae) has also been reported on cotton endophytically colonized by either *B. bassiana* or *Lecanicillium lecanii* R. Zare & W. Gams (Hypocreales: Clavicipitaceae) (Gurulingappa *et al.,* 2010). Another possible role for fungal endophytes could include plant growth promotion as well as impact on tritrophic interaction (Akello *et al.,* 2007; Harish *et al.,* 2008; Vega *et al.,* 2008; Paparu *et al.,* 2009). Fungal entomopathogens that become established as endophytes can therefore play an important role in the regulation of insect populations.

The objectives of this study were to determine whether selected fungal isolates are able to endophytically colonize *V. faba* and *P. vulgaris* plants and to assess their negative effects on the life-history of *L. huidobrensis* following endophytic colonization of the host plant.

3.1 Materials and Methods

3.1.1 Fungal cultures

Ten fungal isolates in six genera, obtained from the International Centre of Insect Physiology and Ecology (*icipe*)'s Arthropod Germplasm Centre, were used in this study: *Beauveria* (3), *Hypocrea* (1), *Gibberella* (1), *Metarhizium* (2), *Trichoderma* (1) and *Fusarium* (2). Eight of the fungal isolates were isolated from aboveground parts of maize, sorghum and Napier grass and were able to endophytically colonize maize and bean seedlings (Akello, 2012). *Beauveria bassiana* isolate ICIPE279 and *Metarhizium anisopliae* isolate ICIPE30 were isolated from Coleopteran larvae and a *Busseola fusca* pupa, respectively. The isolates were cultured on potato dextrose agar (PDA), except *Metarhizium* which was cultured on Sabouraud dextrose agar (SDA), and were maintained at 25 ± 2 ^{o}C in complete darkness. Conidia were harvested by scraping the surface of 2-3-week old sporulating cultures with a sterile spatula. 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. Conidial counts were done using a Neubauer Hemacytometer (Goettel and Inglis, 1997). The conidial suspension was adjusted to 1 x 10^{8} conidia ml^{-1} through dilution prior to inoculation of seeds.

Spore viability was determined before any bioassay by plating 0.1 ml of 3 x10^{6} conidia ml^{-1} 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 ^{o}C and were 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) using the method described by Goettel and Inglis, (1997). Conidia were deemed to have germinated when the length of the germ tube was approximately two times the diameter of the propagule / conidium. Four replicates were used for each isolate.

3.1.2 Plant inoculation and colonization of endophyte isolates

Inoculation was done by soaking seeds of *V. faba* (a local Kenyan Open Pollinated variety) and *P. vulgaris* (Brown Rose Coco) in conidial suspensions titrated at 10^8 ml^{-1} 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 (Schultz *et al.,* 1998). Seeds were transferred into plastic pots (8 cm diameter x 7.5 cm high) containing the planting substrate (mixture of manure and soil 1:5). The substrate was sterilized in an autoclave for 2 hours at 121 ^oC and allowed to cool for 72 hours prior to planting. Five seeds were sowed per pot and maintained at room temperature (25 ± 3 ^oC and 60% RH). Pots were transferred immediately after germination to a screen house (2.8 m length x 1.8 m width x 2.2 m height) at 25 ± 3 ^oC, for two weeks. Seedlings were thinned to three per pot after germination and were watered twice per day (morning and afternoon). No additional fertilizer was added to the substrate.

To determine the colonization of inoculated fungal isolates in *V. faba* and *P. vulgaris*, plants were carefully removed from the pots two weeks after inoculation and were washed with tap water. Seedlings (ca. 30 cm in height) were cut into different sections (ca. 5 cm long): leaves, stems and roots sections. Five randomly selected leaf, stem, and root sections from each plant were surface-sterilized as described above. The different plant parts were then aseptically cut in the laminar flow hood into 1 x 1 cm pieces before placing the pieces, 4 cm apart from each other, on PDA plates amended with a 0.05% solution of antibiotic (streptomycin sulfate salt) (Dingle and McGee, 2003; Istifadah and McGee, 2006; Gurulingappa *et al.,* 2010). Plates were incubated at 25 ± 1 ^oC for 10 days, after which the presence of endophyte was determined. The last rinse water was also plated out to assess the effectiveness of the surface sterilization procedure as described earlier. The colonization of the different plant parts was 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

and Fisher, 1987). Only the presence of endophytes that were inoculated was scored. Slides were prepared from the mother plates and were used for morphological identification. Treatments were randomized in complete block design (RCBD) and the experiment replicated four times over time.

3.1.3 Insects

Liriomyza huidobrensis was obtained from the Animal Rearing and Quarantine Unit, *icipe*. The initial colony originated from adult leafminers collected from wild crucifers on the *icipe* campus (01º13.3′S 36º53.8′E, 1600 m a.s.l.) and was reared on *V. faba* in Plexiglas cages (50 cm x 50 cm x 45 cm) for 8-10 generations prior to experiments. Fly colonies were maintained at 27 ± 2 ºC with a photoperiod of 12L: 12D and relative humidity of approximately 40%.

3.1.4 Effects of endophytically-colonized *V. faba* host plants on "life history" of *L. huidobrensis*

The effect of *V. faba* endophytically-colonized by fungal endophytes on larval and pupal mortality, fly emergence and longevity of *L. huidobrensis* adults was evaluated in inoculated plants. Two-day old mated adult flies (150 individuals at sex ratio of 1: 2 male: female) were exposed for 48 hours to two week-old endophyte-inoculated host plant seedlings in Plexiglas cages (50 cm x 50 cm x 45 cm). Each cage contained five potted plants and represented a treatment and was maintained at 25–27 ^{o}C, at 50–70% RH and 12L: 12D photoperiod. All the treatments were arranged in a randomized complete block design and the experiment replicated five times over time. After 48 hours post-exposure, plants were replaced with fresh non-inoculated seedlings to prevent excessive oviposition and feeding damage by adult flies. The first batch of inoculated plants was maintained until larvae reached the 2nd and 3rd instars (approximately 5–8 days post-exposure). In the control, plants were not inoculated with fungal pathogens. Survival was recorded by counting the number of live flies daily inside the cages until all flies died. Dead flies were placed on Petri dishes lined with damp sterilized filter paper to allow fungal growth on the surface of the cadaver (mycosis test).

Pupae were harvested from leaves 11 days post-exposure, counted and then incubated at 25 ± 1 ^{o}C. Adult emergence and sex ratio were determined. In order to determine adult longevity, 50 adult flies were selected from the above experiment (progenies) and their mortality recorded daily until all flies had died. The flies were maintained in a cage as described above. A 10% sugar solution was provided as food and cages maintained at 25 ±1 ^{o}C.

3.1.5 Statistical analyses

Mortality, number of pupae, emergence and survival data were analyzed using both analysis of variance (ANOVA) and survival analysis techniques. The survival curves were generated using the Kaplan-Meir (K-M) method. The log-rank test was used to compare the effect of various isolates on survival of *L. huidobrensis*.

The K-M estimator of the survivor function was:

$$\widehat{S}(t) = \prod_{y_{(i)} \leq t} \widehat{p_i} = \prod_{y_{(i)} \leq t} \left(\frac{n_i - d_i}{n_i} \right) = \prod_{i=1}^{k} \left(\frac{n_i - d_i}{n_i} \right),$$

where $y_{(k)} \leq t < y_{(k+1)}$, n_i = the number in the risk set just before time t, d_i = number died at time $y_{(i)}$, p_i = probability of surviving through the interval given being alive at the beginning of the interval, and $y(i)$ denotes the ith distinct ordered censored or uncensored observation.

The number of pupae was log-transformed [Log_{10} (x + 1)] before ANOVA analysis while the emergence rate and fungal colonization percentages were square root-transformed [$\sqrt{(x + 1)}$] before applying ANOVA analysis. Tukey HSD multiple comparisons of means were used to separate the means. The success rate (%) of fungal colonization of host plants parts was calculated as follows:

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

All the analyses were performed using R (2.13.1) statistical software (R Development Core Team, 2011) while relying heavily on the epicalc package (Chongsuvivatwong, 2012).

3.2 Results

3.2.1 Endophytic colonization of *P. vulgaris* and *V. faba* by fungal isolates

In viability tests, >90% of conidia of all the isolates germinated. With exception of *M. anisopliae* isolates which failed to colonize both host plant species, all other fungal isolates were able to colonize both host plants. However, colonization of the different parts of the plant (root, stem and leaves) varied depending on fungal isolates and host plants (Figure 3.1).

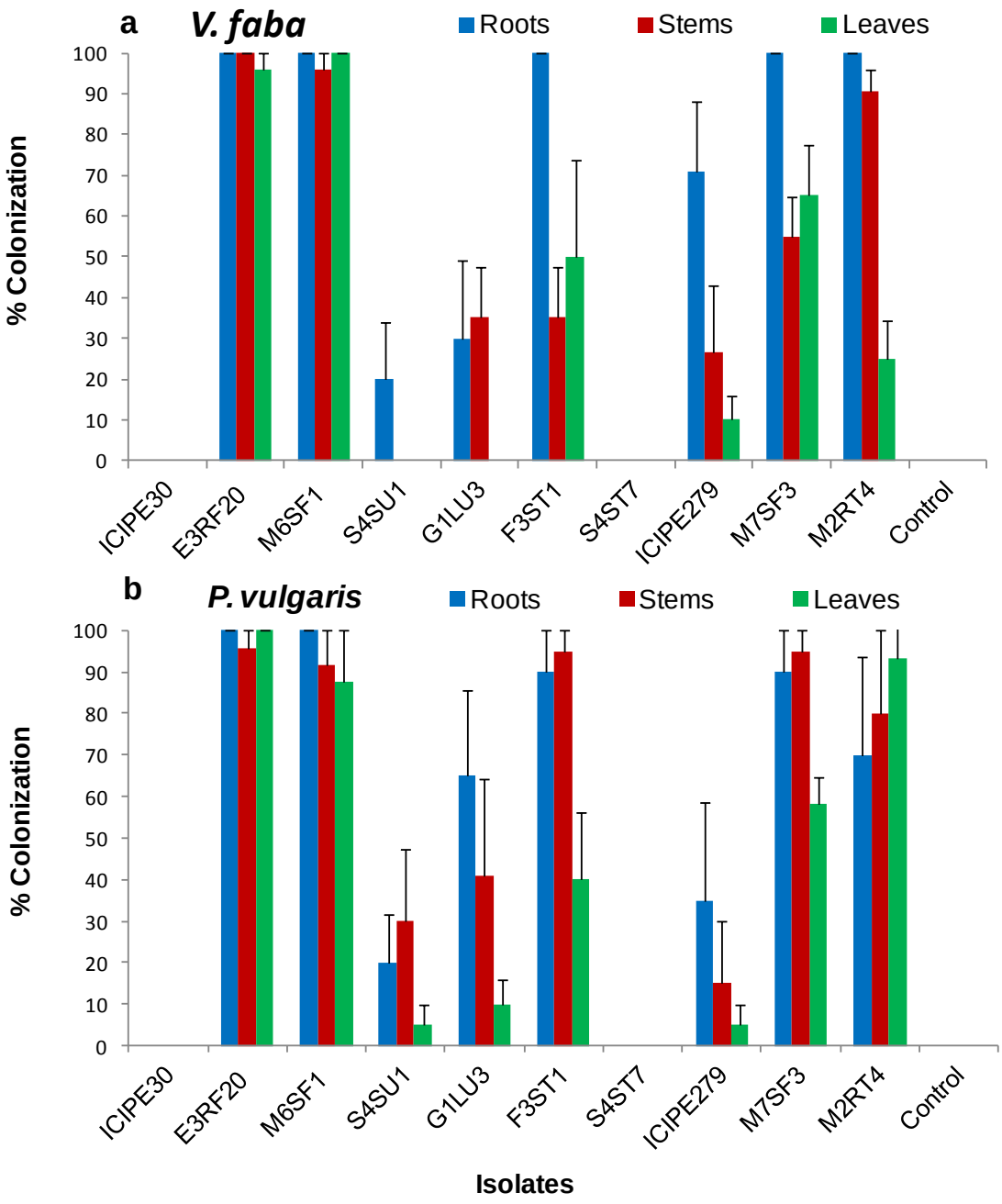

Figure 3.1: Colonization of different parts of *Vicia faba* (*a*) and *Phaseolus vulgaris* (*b*) plants by endophytic isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), *Fusarium oxysporum* (M6SF1 and M7SF3), *Trichoderma asperellum* (M2RT4), *Hypocrea lixii* (F3ST1) and *Gibberella moniliformis* (E3RF20) and non-endophytic isolates of *Metarhizium anisopliae* (ICIPE30 and S4ST7).

For example, isolates of *B. bassiana* ICIPE279, *H. lixii* F3ST1, *G. moniliformis* E3RF20, *F. oxysporum* M6SF1 and M7SF3, and *T. asperellum* M2RT4 colonized roots, stems and leaves of both host plants. *Beauveria bassiana* G1LU3 and S4SU1 colonized the roots, stems and leaves of *P. vulgaris* plants while isolate G1LU3 colonized only roots and stems of *V. faba* and S4SU1 only the roots (Figure 3.1). There were however significant differences in percentage colonization between the two host plants and fungal isolates (Figure 3.1). For instance, *G. moniliformis* E3RF20 and *F. oxysporum* M6SF1 colonized more than 90% of the plant parts sampled of both host plants, whereas *B. bassiana* G1LU3 colonized 60, 40 and 10% of roots, stems and leaves of *P. vulgaris*; and 30 and 35% of roots and stems of *V. faba*, respectively. *Fusarium oxysporum* M7SF3 successfully colonized 90, 95 and 58.4% of roots, stems and leaves of *P. vulgaris,* respectively, and 100, 55 and 65% of roots, stems and leaves in *V. faba*, respectively (Figure 3.1). There was a significant interaction between fungal isolates and *V. faba* plant parts ($F = 4.159$, $df = 20, 99$, $p < 0.001$), implying that isolates colonized differently roots, stems and leaves of the plant. For instance, significant differences in colonization by isolates were observed on roots ($F = 26.81$, $df = 10, 33$, $p < 0.0001$), stems ($F = 24.65$, $df = 10, 33$, $p < 0.0001$) and leaves ($F = 19.99$, $df = 10, 33$, $p < 0.0001$) of *V. faba* (Figure 3.1). Although there was no interaction between fungal isolate-plant parts ($F = 1.191$, $df = 20, 99$, $p < 0.278181$) in *P. vulgaris,* there were differences in colonization levels of each fungal isolates ($F = 35.6$, $df = 10, 99$, $p < 0.0001$) and plant parts ($F = 7.14$, $df = 2, 99$, $p = 0.0012$), and colonization of plant parts: roots ($F = 10.71$, $df = 10, 33$, $p < 0.0001$), stems ($F = 12.23$, $df = 10, 33$, $p < 0.0001$), and leaves ($F = 18.55$, $df = 10, 33$, $p < 0.0001$) (Figure 3.1).

3.2.2 Effect of endophytically-colonized *V. faba* host plant on life- parameters of *L. huidobrensis*

3.2.2.1 *L. huidobrensis* adult survival

The longevity of the first generation of *L. huidobrensis* adults exposed to different plant parts of *V. faba* endophytically-colonized was significantly reduced ($p < 0.0001$) as compared to the control (Figure 3.2). Mortality of 100% was observed in *L. huidobrensis*

within 13.2 ± 0.7 and 15 ± 0.6 days (*F = 29.84; df = 8, 40, p<0.0001*) as compared to 27.3 ± 1.1 days in the controls (Figure 3.2).

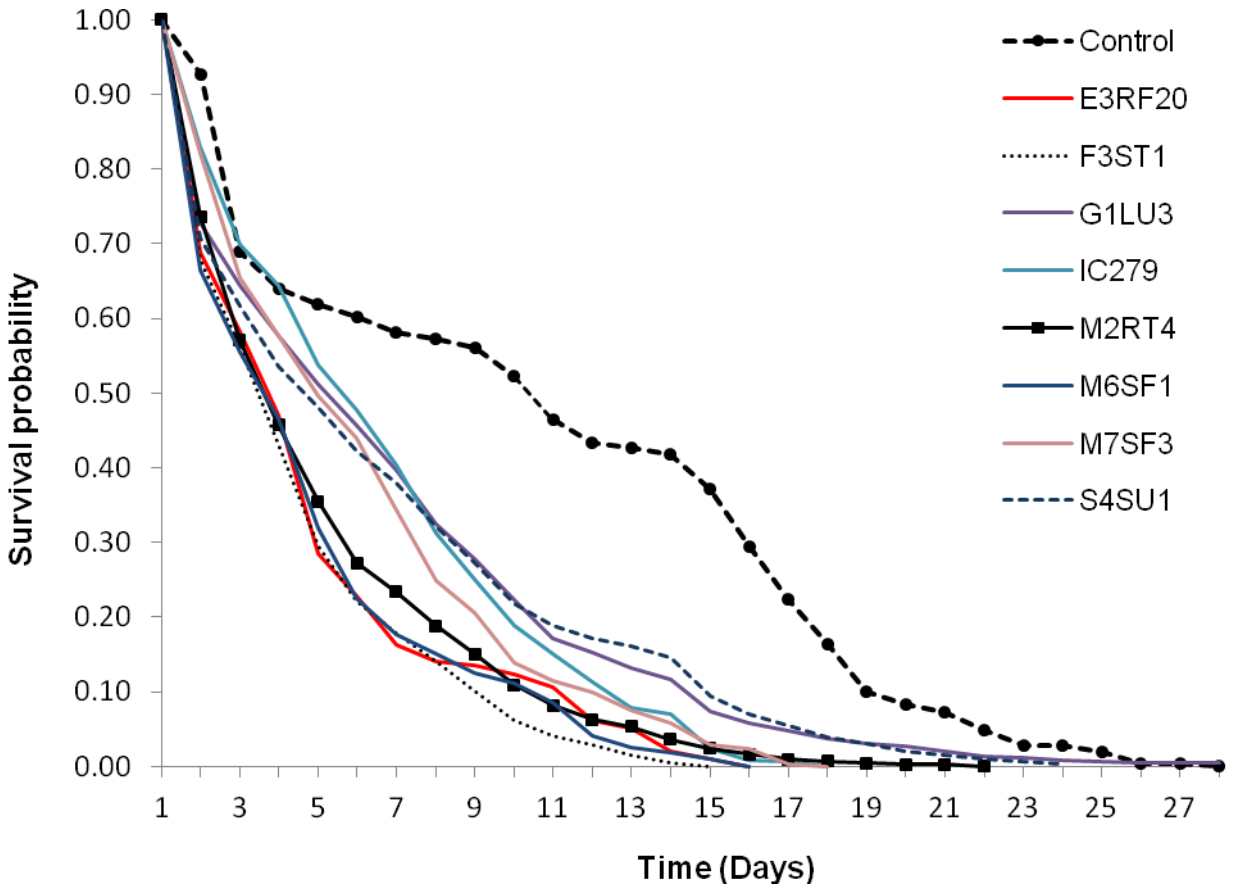

Figure 3.2: Survival curves for *Liriomyza huidobrensis* adults following exposure to *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), *Fusarium oxysporum* (M6SF1 and M7SF3), *Trichoderma asperellum* (M2RT4), *Hypocrea lixii* (F3ST1) and *Gibberella moniliformis* (E3RF20) two weeks prior to exposure.

The survival of adult flies exposed to endophytically-inoculated plants varied among the treatments (*proximate log rank test = 587.6, df = 8, p < 0.001*). For example, at 5 days post-exposure, mean survival was 22.2% with *H. lixii*, 42.3% with *B. bassiana* S4SU1 and 64% in the control (Figure 3.2). High mortality of adult *L. huidobrensis* was

observed over the first 5 days post-inoculation (Figure 3.2). At 10 days post-exposure, mean adult survival ranged between 4.2 and 18.9% in all the fungus treatments as compared to 56.4% in the control (Figure 3.2). At day 20 post-exposure, the mean survival in all the treatments including the control was low. No adult survival was observed in any of the fungal treatments, except with *B. bassiana* S4SU1 (1.4% survival) and G1LU3 (2.0% survival) (Figure 3.2).

The longevity of flies exposed to endophytically-inoculated plants ranged between 13.2 ± 0.7 and 15.0 ± 0.6 days as compared to 27.3 ± 1.1 days in the control ($F = 29.84$; $df = 8, 40$, $p<0.0001$). Among the fungal isolates, *H. lixii* F3ST1 outperformed the other isolates ($F = 23.04$; $df = 8, 40$, $p<0.0001$ at 95% CI) in reducing mean adult longevity and adult survival (Table 3.1).

Table 3.1: Mean survival period (longevity) of *Liriomyza huidobrensis* adults reared on *Vicia faba* plants colonized by isolates of different endophytic fungi

Fungal species	**Isolates**	**Mean survival time (Days) ± SE**
Beauveria bassiana	ICIPE279	5.79 ± 1.46 d
Beauveria bassiana	G1LU3	6.06 ± 1.21 c
Beauveria bassiana	S4SU1	5.97 ± 1.23 cd
Fusarium oxysporum	M7SF3	5.32 ± 1.33 f
Fusarium oxysporum	M6SF1	3.97 ± 1.06 a
Gibberella moniliformis	E3RF20	4.06 ± 1.18 a
Hypocrea lixii	F3ST1	3.75 ± 1.03 b
Trichoderma asperellum	M2RT4	4.37 ± 1.10 e
Control	–	9.89 ± 1.89 g

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

3.2.2.2 *L. huidobrensis* pupation

Fewer *L. huidobrensis* pupae were produced in endophytically-colonized plants than in the control (387.0 ± 21.7 pupae) (Figure 3.3). There were, however, significant differences ($F = 9.3$, $df = 8, 40$, $p < 0.0001$) between the fungal isolates. For example, the number of pupae produced in *G. moniliformis* E3RF20 (288.0 ± 40.1) was not significantly different from the control but was different from the other fungal isolates. *Hypocrea lixii* F3ST1-infected plants produced the least number of pupae (80.0 ± 6.7), followed by plants inoculated with *B. bassiana* S4SU1 and G1LU3 with 118.0 ± 13.9 and 124.0 ± 11.6 pupae, respectively (Figure 3.3).

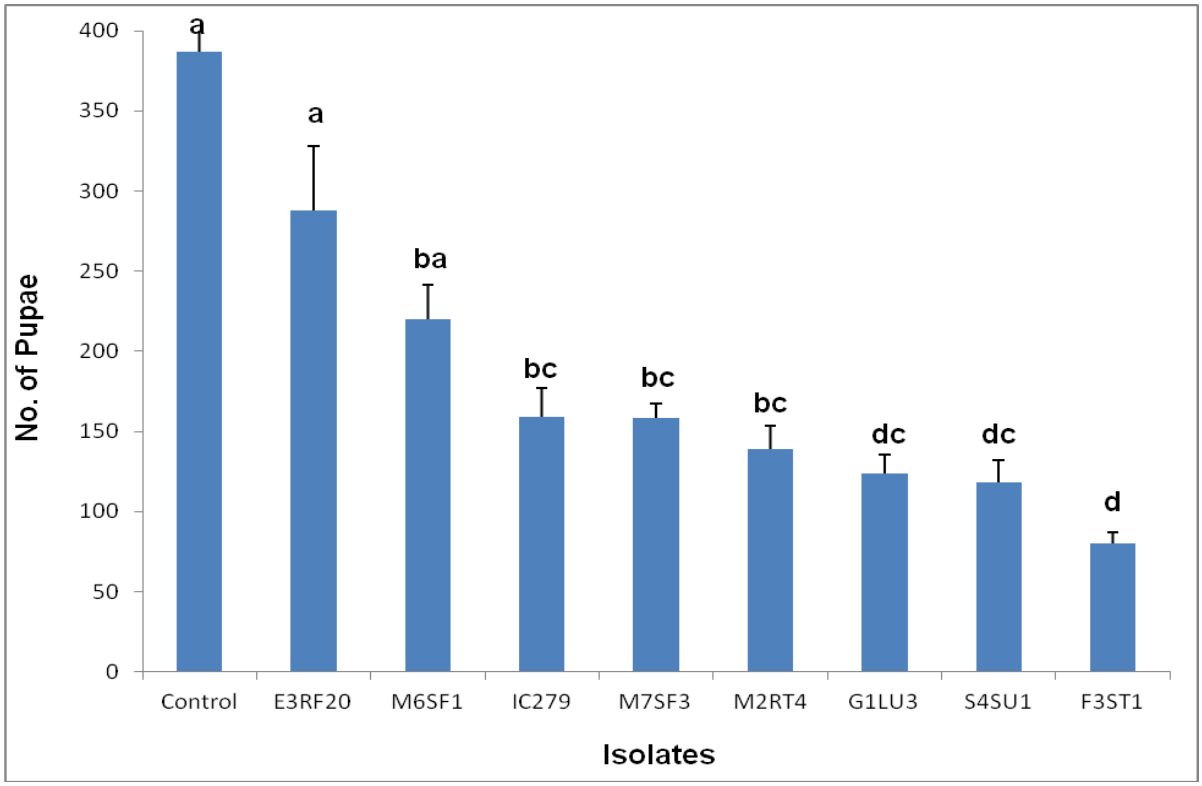

Figure 3.3: Effect of exposure to *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), *Fusarium oxysporum* (M6SF1 and M7SF3), *Trichoderma asperellum* (M2RT4), *Hypocrea lixii* (F3ST1) and *Gibberella moniliformis* (E3RF20) on the number of pupae produced by *Liriomyza huidobrensis*. Bars denote means ± one standard error at 95% CI (p = 0.05).

3.2.2.3 Adult emergence

Higher numbers of adult flies emerged from control plants (82.9 ± 7.6%) than from endophytically-colonized plants (Figure 3.4). However, there were significant differences (*F = 14.7*, *df = 8*, 70, *p< 0.0001*) in the numbers of adult fly emergence among the fungal isolates (Figure 3.4). Few flies emerged from pupae of plants endophytically-colonized by *H. lixii* F3ST1 (38.0 ± 2.9%) and *B. bassiana* G1LU3 (21.4 ± 3.7%) as compared to the ones colonized by *G. moniliformis* E3RF20 (33.6 ± 4.5%) and *F. oxysporum* M6SF1 (40.8 ± 4.1%) (Figure 3.4).

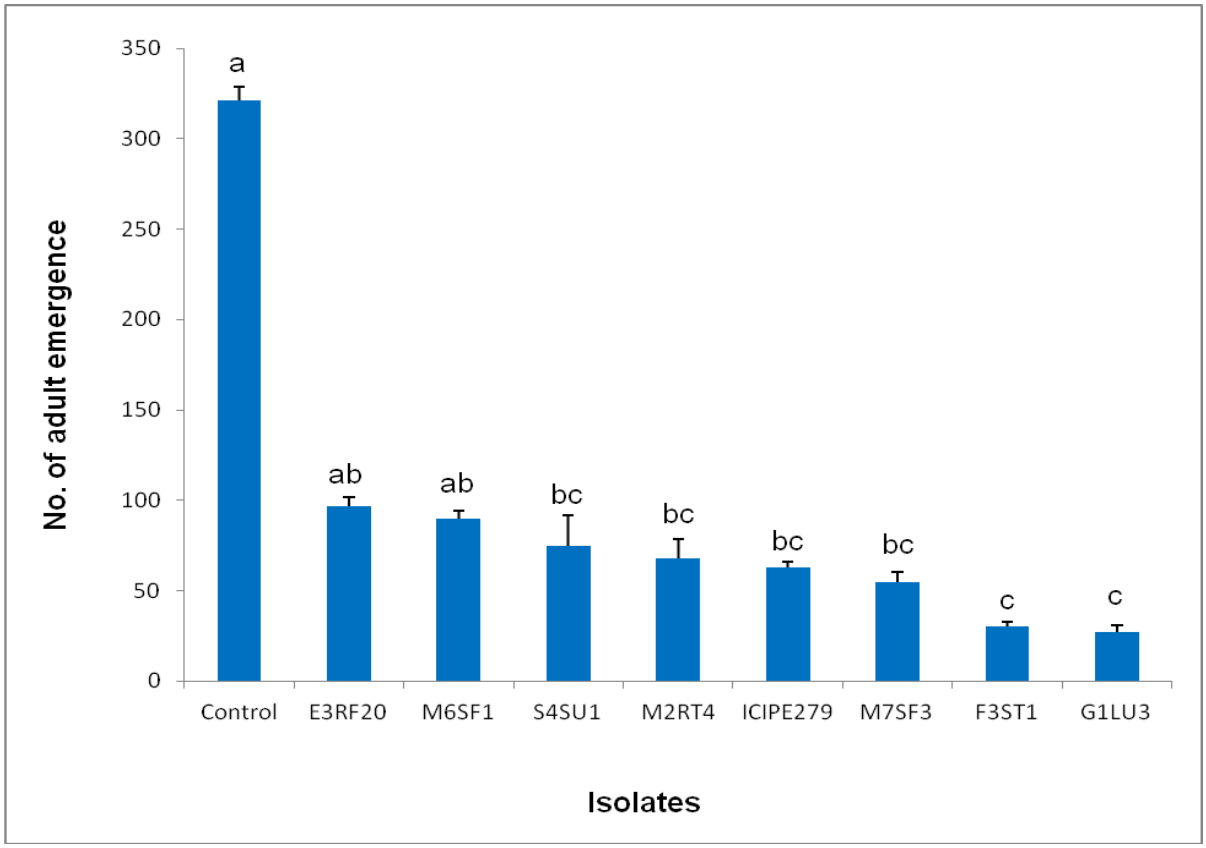

Figure 3.4: Effect of exposure to *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), *Fusarium oxysporum* (M6SF1 and M7SF3), *Trichoderma asperellum* (M2RT4), *Hypocrea lixii* (F3ST1) and *Gibberella moniliformis* (E3RF20) on adult emergence of *Liriomyza huidobrensis*. Bars denote means ± one standard error at 95% CI (p = 0.05).

Most flies in the control succeeded to emerge while flies from fungus-inoculated plants failed to emerge because they either got stuck (whole or part of the body) in the pupal case or died inside the pupae (Figure 3.5). This phenomenon was most pronounced in *Beauveria, Fusarium* and *Hypocrea*-inoculated *V. faba* plants. In the case of *Fusarium*-inoculated plants, the whole abdomen as well as hind and mid legs of the body parts were stuck inside the pupal case while in *H. lixii*-treatment only the hind legs were stuck inside the case (Figure 3.5).

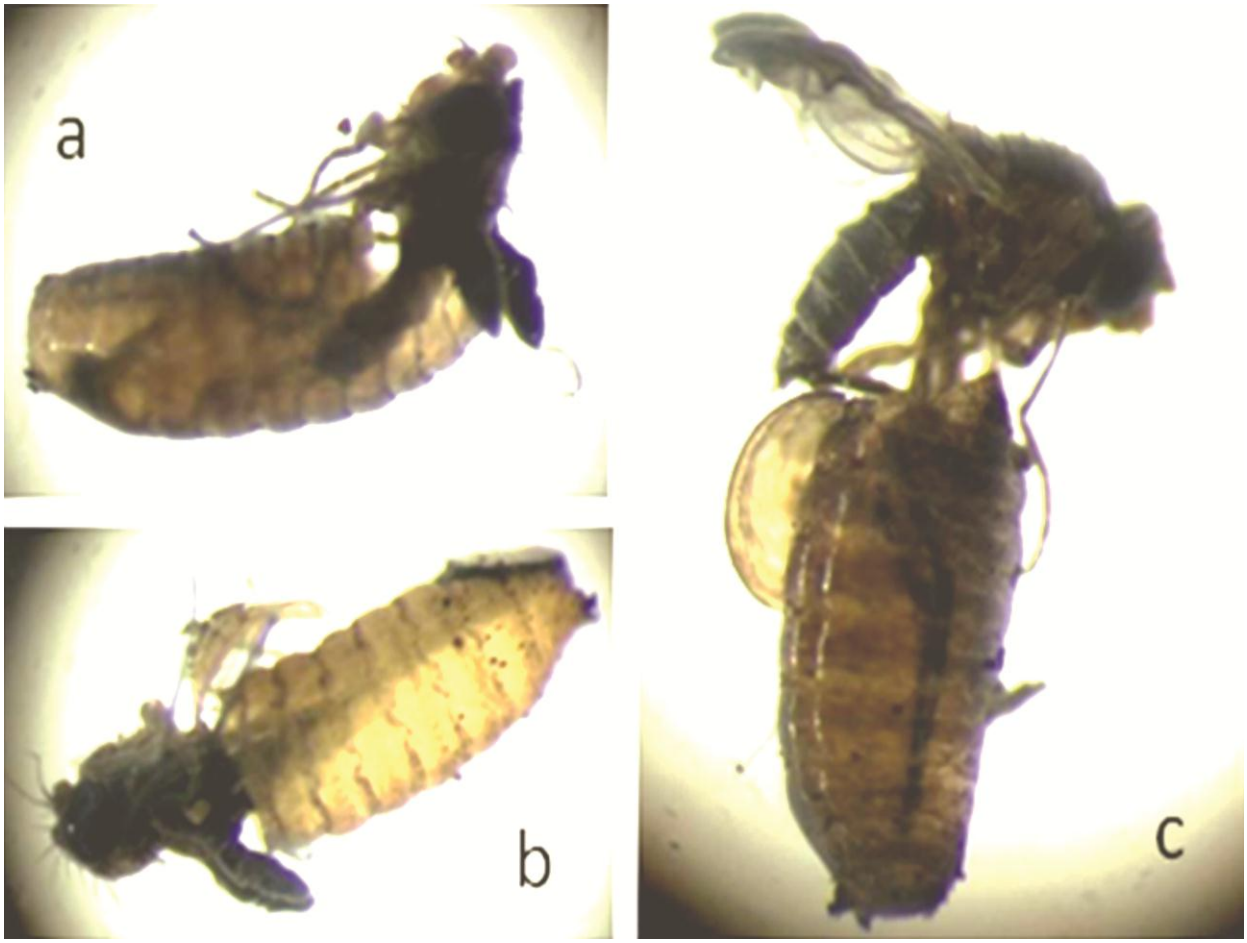

Figure 3.5: Effect of *Beauveria* (a)*, Fusarium* (b) and *Hypocrea* (c) isolates on the *L. huidobrensis* emergence (adult insects got stuck inside the pupal skin when emerging).

3.2.2.4 Sex ratio

There was a significant difference (*F = 3.2, df = 8, 70, p < 0.0042*) in sex ratio between males and females among the treatments. The difference in numbers of males and females was positive (Males–Females = 0.5619724) with a *p-value* = 0.00007, indicating that more males emerged than females in most of the treatments as compared to the control. For instance, the sex ratio was (1 : 1.08), (1 : 1.29) and (2 : 1) (males: females) in the control, *T. asperellum* M2RT4 and *B. bassiana* S4SU1 treatments, respectively (Table 3.2).

Table 3.2: Sex ratio (Males – Females) of *Liriomyza huidobrensis* individuals that emerged from endophytically-colonized *Vicia faba* plants

Fungal species	**Isolates**	**Sex ratio (Males : Females)**
Beauveria bassiana	ICIPE279	1.24 : 1
Beauveria bassiana	G1LU3	1.40 : 1
Beauveria bassiana	S4SU1	1.46 : 1
Fusarium oxysporum	M7SF3	1.27 : 1
Fusarium oxysporum	M6SF1	1.21 : 1
Gibberella moniliformis	E3RF20	1.27 : 1
Hypocrea lixii	F3ST1	1.27: 1
Trichoderma asperellum	M2RT4	1 : 1.29
Control	–	1 : 1.08

3.2.2.5 Effect of endophytic fungal isolates on survival of the progeny

The survival curves of the progeny from flies whose parents were previously exposed to inoculated plants differed significantly among the treatments (*proximate log rank test = 117.9, df = 8, p < 0.001*) (Figure 3.6).

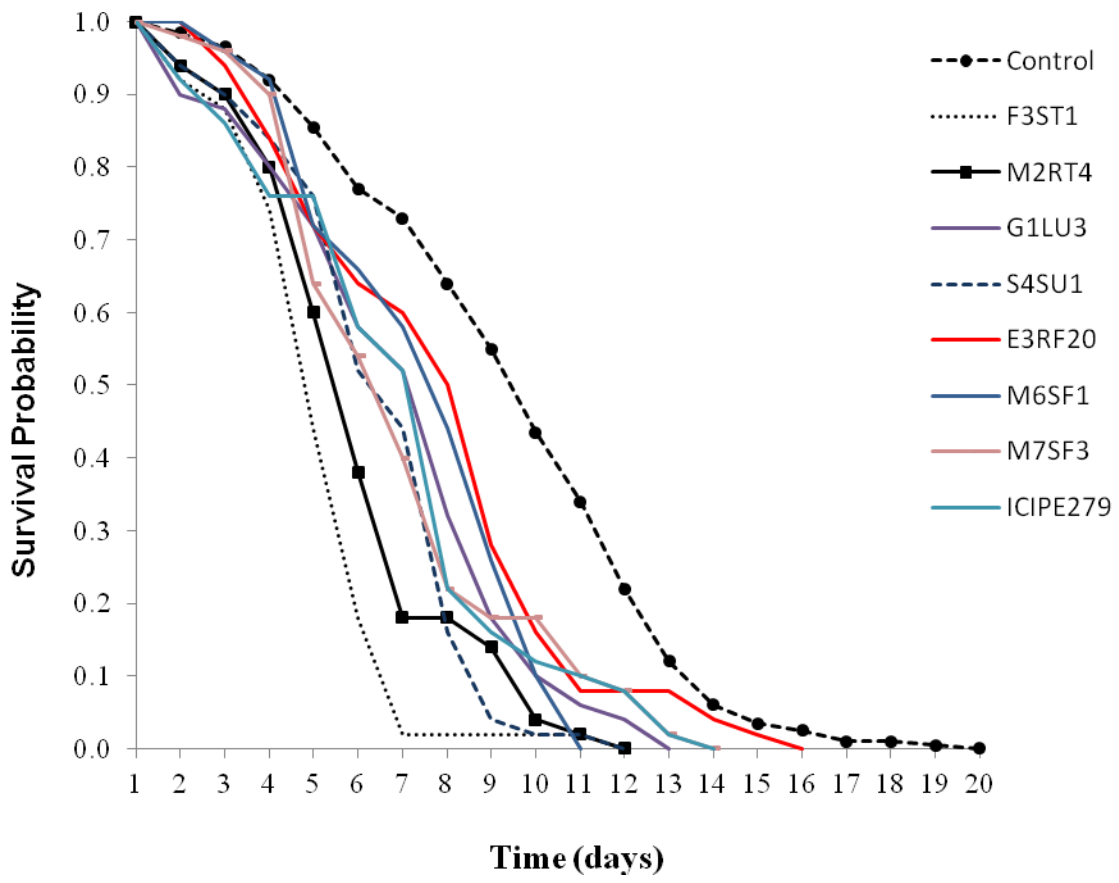

Figure 3.6: Progeny longevity curves of *Liriomyza huidobrensis* emerging from *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), *Fusarium oxysporum* (M6SF1 and M7SF3), *Trichoderma asperellum* (M2RT4), *Hypocrea lixii* (F3ST1) and *Gibberella moniliformis* (E3RF20).

For instance, at 7 days post-emergence, only 2% survival was recorded with *H. lixii* F3ST1 and between 18 and 50% with the other isolates in contrast to 73% in the control. At 10-day post-emergence, 2-18% survival was observed in all the fungal treatments as compared to 57.5% in the control (Figure 3.6). At 14 day, no survival was observed in any of the fungal treatments, except with *G. moniliformis* E3RF20 (4% survival) (Figure 3.6).

The longevity of flies exposed to *H. lixii* F3ST1 was the shortest (11.2 ± 1.0 days), followed by *B. bassiana* S4SU1 (11.8 ± 1.6 days), *T. asperellum* M2RT4 (12.0 ± 1.0 days) as compared to the control (17.8 ± 1.4 days). There was a significant difference ($p<0.001$) between the average survival period of the parent insects exposed to *H. lixii* F3ST1 inoculated plants (5.3 days) and those that emerged (progeny) from the *Hypocrea*-inoculated plants (3.8 days).

No mycosis was observed among all the 6750 cadavers exposed to endophytically-colonized *V. faba* plants.

3.3 Discussion

Colonization of the two host plant species varied according to fungal isolate, host plant and different parts of the host plant. Similar results were reported by Gurulingappa *et al.* (2010) with *B. bassiana* and *A. parasiticus. Beauveria bassiana* was able to colonize entire wheat plants but not cotton, while *A. parasiticus* colonized different plant parts of wheat and cotton. Akello (2012) also reported that *B. bassiana* S4SU1, *G. moniliformis* E3RF20, *T. asperellum* M2RT4 and *M. anisopliae* S4ST7 (the same isolates used in the present study) were able to colonize maize and bean plants. In the present study, isolates of *M. anisopliae* failed to colonize both *V. faba* and *P. vulgaris,* which contrasts with the findings of Akello (2012) who reported endophyte colonization of maize and beans by *M. anisopliae.* There could then be different levels of hosts and tissues specificity within a genus/species. There were differences in level of colonization of different parts of host plant among fungal isolates. Similar results were reported by Gurulingappa *et al.* (2010), Akello (2012), Posada *et al.* (2007) and Vega *et al.* (2008).

Since *M. anisopliae* isolates ICIPE30 and S4ST7 did not colonize the sampled tissues of *V. faba* or *P. vulgaris,* they can be considered as non-endophytic on these two host plants. However, this classification has to be taken with caution and clearly stresses the need to include host plant/cultivar detail in the classification. For instance, *M. anisopliae* S4ST7 which was non-endophytic in our study was previously reported to be endophytic

on sorghum, maize and Napier grass (Akello, 2012). Recently, Sasan and Bidochka, (2012) also reported endophytic colonization of *Panicum virgatum* L. (Poaceae) and *P. vulgaris* by *Metarhizium robertsii*. The different observations made on the endophytic capability of *M. anisopliae* S4ST7 on the two bean species used in our study and that of Akello, (2012) indicates that endophytic abilities of particular isolates may be strongly associated with host plant species as well as species varieties (different bean varieties used in this study).

All the fungal isolates that endophytically-colonized *V. faba* were also pathogenic to *L. huidobrensis,* causing 100% mortality. Similar results were reported by Vega *et al.* (2008) on adult coffee berry borers, *Hypothenemus hampei* (Coleoptera: Scolytidae) with endophytic *B. bassiana* and *Clonostachys rosea* (Hypocreales: Bionectriaceae) and Akello *et al.* (2008b) on *C. sordidus* with *B. bassiana. Hypocrea lixii* F3ST1 and *B. bassiana* G1LU3 significantly reduced the number of pupae produced by female *L. huidobrensis* and their emergence from pupal skins. Similar results were reported by Akello, (2012) on *L. trifolii* and *L. sativae* with *B. bassiana.* In addition to pupal mortality, fungal endophytes reduced survival of the *L. huidobrensis* adults, which corroborates the findings of Akello *et al.* (2008a) who reported reduction in larval survival of *C. sordidus* following colonization of banana by *B. bassiana*. Vega *et al.* (2008) also observed a reduction in survival of adult *H. hampei* treated with *B. bassian*a and *C. rosea.* Entomopathogens as endophytes can also slow down reproduction rate (longer time to reproduction and fertility), reduce feeding, growth and fecundity of insects (Gurulingappa *et al.,* 2010). In the present study both *B. bassiana* G1LU3 and *H. lixii* F3ST1 had the largest negative effect on emergence of *L. huidobrensis* while *H. lixii* F3ST1 had the largest overall effect on insect population and can be considered as promising biocontrol candidates for the management of *L. huidobrensis* populations.

More males emerged from fungus-infected host plants than uninfected plants. This is an indication that most of the eggs laid by the females were not fertilized (Taylor and Yuval, 1999; Ward, 2000). Female insects choose to fertilize fewer eggs in the presence of the endophytes inside the plant, thus, affecting the oviposition ability/pupation of the *L.*

huidobrensis. In most insect species, reproduction involves long-term storage of sperm in the spermatheca. Spermatozoa are released progressively from the spermatheca to fertilize oocytes or eggs (Thornhill and Alcock, 1983; Taylor and Yuval, 1999). In cases where female insects experience limited resources or are in danger or under no-choice conditions (like in presence of metabolites produced by plants endophytically), they might simply avoid laying eggs or lay few eggs without fertilizing them (Charnov and Skinner, 1985). This phenomenon was also observed in dung flies and Mediterranean fruit flies (Taylor and Yuval, 1999; Ward, 2000). Additionally, the cryptic females choice might also be due to females response to plant growth and plant quality as described by Craig *et al.* (1992), where being male-biased could be response to plant growth endophytically-colonized and progeny sex ratio varied in response to plant quality. There are then unequal returns on investment in male and female progeny. The progeny sex ratio can then be related to endophytically-colonized *V. faba* plant growth and its quality (Craig *et al.,* 1992).

The life cycle of the F1-generation that emerged from *V. faba* plants inoculated with *H. lixii* F3ST1, *B. bassiana* S4SU1 and *T. asperellum* M2RT4 was shorter than one of the other isolates and control. Vega *et al.* (2008) reported similar effects of an endophytic *B. bassiana* SPCL 03047 on adult *H. hampei* with an average survival period of 4.8 ± 0.2 days compared to 15.0 ± 0.6 days for uninoculated plants.

The fact that no mycosed insects were recorded suggests that adult mortality of *L. huidobrensis* could be as a result of feeding deterrence or antibiosis (Lewis and Cossentine, 1986; Lewis and Bing, 1991; Bing and Lewis, 1991; Bing and Lewis, 1992ab; Lewis *et al.,* 1996; Cherry *et al.,* 2004; Vega *et al.,* 2008). The mechanism by which endophytic fungi interact with insects is still unclear but one can speculate on production of metabolites/antibiosis (Vega *et al.,* 2008). Lewis and Bing, (1991) and Bing and Lewis, (1991, 1992ab) suggested that the reduced tunneling of *Ostrinia nubilalis* (Lepidoptera: Crambidae) following endophyte colonization of maize by *B. bassiana* could be due to the presence of fungal metabolites that cause feeding deterrence or antibiosis. This might have been explained by the absence of *B. bassiana*

infection (mycosis) within *O. nubilalis* individuals that fed on endophytically-infected plants. Studies by Cherry *et al.* (2004) on *Sesamia calamistis* (Lepidoptera: Noctuidae) also support the feeding deterrence/antibiosis hypothesis since larvae feeding on plants injected with *B. bassiana* were smaller than those in the control plants.

In addition, these results also confirmed that there may be possibility of an indirect effects, induced systemic resistance (ISR) elicited by the presence of endophytes especially *H. lixii* and *B. bassiana* on the developing seedlings. According to Hell and Bostock (2002), many plants respond to local attack by herbivores or pathogens with a de novo production of compounds reducing or inhibing further attack by, or performance of their enemies. Responses occurred both in plant organ originally attacked (local response) and in distant, yet unaffected parts (systemic response). One of these responses is induced systemic response (ISR, or systemic acquired resistance SAR) of plants against pathogens (Hunt *et al*., 1996; Schneider *et al*., 1996; Sticker *et al*., 1997; Mauch-Mani and Métraux, 1998; Hammerschmidt, 1999; Hell and Bostock, 2002; Bargabus *et al*., 2004). It was noted that even when only a part or all the parts of the host *V. faba* plant were endophytically-colonized by a fungus, there was a significant reduction in *L. huidobrensis* survival, number of pupae produced and adult emergence. This confirmed the effects of ISR reported by Hell and Bostock (2002) against pathogens in the context induced plant defences, and Bargabus *et al*. (2004) in sugar beet.

3.4 Conclusion

Of the ten fungal isolates screened, eight presented endophytic properties. Each of these isolates colonized both *V. faba* and *P. vulgaris*, suggesting that they could also be potential endophytes on other Fabaceae plants, although this would need to be confirmed. Further studies are warranted to identify the range of *Liriomyza* spp. host plants in which endophytic properties could be expressed by these isolates. The two *M. anisopliae* isolates did not colonize any of the two host plant species. *Hypocrea lixii* F3ST1 and *B. bassiana* (ICIPE279, S4SU1 and G1LU3) were the most virulent isolates

against *L. huidobrensis* in causing mortality, reducing adult survival, number of pupae, progeny survival and adult emergence. The endophytic properties and the negative effects on host insect of the promising isolates identified in this study warrant further research into their mode of action, development and use in the management of *L. huidobrensis.* It was accidentally discovered that these promising fungal endophytes isolates have negative effects on red spider mites (*Tetranychus evansi* and *T. urticae*) when inoculated into *V. faba* (Annex 1) and can also control them. Studies are still undergoing for more investigation on the effects of these endophytes on red spider mites.

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CHAPTER 4: INTERACTIONS BETWEEN PHAEDROTOMA SCABRIVENTRIS NIXON (HYMENOPTERA: BRACONIDAE) AND DIGLYPHUS ISAEA WALKER (HYMENOPTERA: EULOPHIDAE), PARASITOIDS OF THE PEA LEAFMINER LIRIOMYZA HUIDOBRENSIS (BLANCHARD) (DIPTERA: AGROMYZIDAE)

Abstract

Liriomyza leafminer flies represent a serious threat to horticultural production in East Africa. Field parasitism rates recorded in Kenya were below 5%, with *Diglyphus isaea* Walker being one of the predominating parasitoid species in the country. *icipe*, in collaboration with the International Potato Centre (CIP), imported into Kenya the endoparasitoid *Phaedrotoma scabriventris* Nixon to improve on natural control of the pest. Under laboratory conditions, while used separately, parasitism rates of *D. isaea* and *P. scabriventris* were 30.4 ± 10.9% and 63.6 ± 7.7% respectively. However, while used simultaneously, the parasitism rate increased up to 77.0 ± 5.3%. There were no superparasitism or hyperparasitism observed between the two parasitoids. In addition, both parasitoids induced leafminer mortality through larval-feeding and stinging. The larval feeding-stinging mortality was higher with the ectoparasitoid *D. isaea* (41.9 ± 9.1%) compared to the endoparasitoid *P. scabriventris* (11.9 ± 8.7). Similarly, pupal mortality due to feeding-stinging activity was 49.1 ± 6.5% and 21.6 ± 1.9% when exposed to *D. isaea* and *P. scabriventris* respectively. The implication for simultaneous use of both parasitoids in East Africa is discussed.

4.0 Introduction

The growth of the horticultural industry in Kenya is limited by a number of pests notably arthropods. Among these pests, are the invasive leaf miner species: *Liriomyza huidobrensis* (Blanchard), *L. sativae* Blanchard and *L. trifolii* (Burgess) (Diptera: Agromyzidae) that pose the greatest challenge as they damage major vegetable and ornamental crops, serve as vector for diseases and constitute quarantine pests in European markets (Johnson *et al*., 1983; Parrella *et al*., 1984; Deadman *et al.,* 2002;

Chabi-Olaye *et al*., 2008; EPPO, 2009). The management of leafminers worldwide, and particularly in East Africa, has commonly relied on the routine use of synthetic chemical insecticides (Chandler, 1981, 1984; Gitonga *et al*., 2010). However, the indiscriminate and frequent use of these chemicals resulted in insecticide resistance of flies (Parella et al., 1984; Murphy and LaSalle, 1999), pollution of the environment as well as elimination of their natural enemies (Johnson *et al*., 1980). Chemical control is also not very effective since flies usually escape insecticide applications due to their high mobility. Furthermore, *Liriomyza* larvae are inaccessible to many pesticides because they develop inside leaves and pupate in soil (Mujica and Kroschel, 2011). Horticultural producers are also under pressure since the introduction of maximum residue level (MRL) set up by the European Union on export produce.

Biological control using parasitoids and entomopathogenic fungi is being considered as alternative to leafminer management in East Africa (Migiro *et al*., 2010). Surveys in Kenya between 2005 and 2007, indicated the presence of various indigenous leafminer parasitoid species, in the order of most to least important *Opius dissitus* Muesebeck (Hymenoptera: Braconidae), *D. isaea*, *Neochrysocharis formosa* (Westwood) (Hymenoptera: Eulophidae) and *Hemiptarsenus varicornis* (Girault) (Hymenoptera: Eulophidae) (Chabi-Olaye *et al*., 2008). However, their parasitism rate was below 5%. It is in this context that the koinobiont parasitoid, *Phaedrotoma scabriventris* Nixon (Braconidae: Opiinae), was introduced into *icipe*'s quarantine facilities in Kenya in 2008 to improve parasitism rates achieved by the indigenous natural enemy complex in East Africa. *Phaedrotoma scabriventris* is the most important leafminer parasitoid in Argentina, Brazil and Peru where it has been reported to cause mortality of between 20 and 52% (Valladares *et al*., 1999, 2001; Kroschel, 2008). In Kenya, the three important *Liriomyza* species (*L. huidobrensis*, *L. sativae* and *L. trifolii*) were found acceptable and suitable for the development of *P. scabriventris* although the parasitoid preferred *L. huidobrensis* (Chabi-Olaye *et al*., 2013). *Phaedrotoma scabriventris* is therefore considered for release against leafminer flies in the horticulture production systems in East Africa. Since *D. isaea* is one of the most important local parasitoids found in Kenya, Uganda and Tanzania (Chabi-Olaye *et al*., 2008; unpublished data),

understanding the interactions between this parasitoid and the exotic *P. scabriventris* is of paramount importance. A range of interactions have been described between parasitoids and include exploitative competition, interference competition with priority effects, cleptoparasitism, facultative hyperparasitism, obligate hyperparasitism, complementarity, additive and synergetic effects (Mills, 2003). In addition to understanding the dynamics of interactions for the selection of effective biocontrol agents, the study will also contribute to evaluating the environmental risks posed by potential direct and indirect non-target impacts from the introduced natural enemies (Mackauer, 1990; Follett and Duan, 1999; Cusumano *et al*., 2011). The objective of the present study was therefore to investigate the interactions between the local ectoparasitoid *D. isaea* and the introduced endoparasitoid *P. scabriventris* in the prospect of using them together for the biological control of *Liriomyza* species in horticultural production systems in East Africa.

4.1 Materials and methods

4.1.1 Host plants

A Kenyan open pollinated variety of Faba bean, *Vicia faba* Linnaeus (Fabales: Fabaceae) was used for the rearing of *L. huidobrensis*. Seeds were planted in plastic pots (8 cm diameter x 7.5 cm high) filled with the planting substrate (mixture of soil and manure 5:1 in a ratio). Pots were maintained in a screen-house (2.8 m length x 1.8 m width x 2.2 m height) at 25 ± 3 ^{o}C for two weeks. Two week-old plants were used for exposure to adult *L. huidobrensis*.

4.1.2 Insects

Liriomyza huidobrensis

Liriomyza huidobrensis was obtained from the Animal Rearing and Quarantine Unit (ARQU), of International Centre of Insect Physiology and Ecology (*icipe*). The colony originated from adult leafminers collected from wild crucifers on the *icipe* campus (01º13.3′S 36º53.8′E, 1600 m a.s.l.) and was reared on *V. faba* for 8 - 10 generations

prior to experiments. Rearing colonies were maintained at 27 ± 2 ºC with a photoperiod of 12L: 12D and relative humidity of approximately 40%. *L. huidobrensis* adults were fed on a 10 % sucrose solution. In order to obtain leafminer-infested plants with larvae of the appropriate size (2nd and 3rd instars), 200 two day-old *L. huidobrensis* adults (sex ratio 1: 2 males: females) were exposed for 24 hours to 10 potted plants in Plexiglas cages (50 cm × 50 cm × 45 cm). Plants were removed and transferred to another similar cage free of adult leafminers to allow development of larvae until the 2nd to 3rd instar (5 – 8 days post-exposure). The exposed-infested potted plants with 2nd and 3rd instar larvae were used for the experiments. Prior to exposure, adult *L. huidobrensis* were fed on 10% sucrose as described above.

Diglyphus isaea

The ectoparasitoid *D. isaea* used in the experiments was also obtained from the ARQU, *icipe*. The colony originated from adult *D. isaea* collected from leafminer-infested French bean, tomatoes and crucifers at Naivasha (S: 00.66731^{o}; E: 036.38603^{o}; Elevation: 1906 m), Kenya. *D. isaea* was reared on *L. huidobrensis*-infested *V. faba* in Plexiglas cages (50 cm x 50 cm x 45 cm) for 5 - 10 generations prior to experiments. For the rearing of parasitoids, *D. isaea* adults were exposed to 2nd and 3rd-instar larvae of *L. huidobrensis* and the colony was maintained at 27 ± 2 ºC with a photoperiod of 12L: 12D and 40-50% rh. . Parasitoids were fed on 10% honey during their given mating period, 24 hours post-emergence.

Phaedrotoma scabriventris

The initial colony of *P. scabriventris* originated from Peru and was maintained in the quarantine facilities of *icipe* on 2nd and 3rd instars *L. huidobrensis* larvae-infested *V. faba* host plants for 5 - 10 generations prior to experiments .Parasitoids were fed on 10% honey during their given mating period, 24 hours post-emergence.

4.1.3 Interactions between *Phaedrotoma scabriventris* and *Diglyphus isaea*

Single, simultaneous and sequential releases

Interactions between *P. scabriventris* and *D. isaea* when parasitizing *L. huidobrensis* were studied following the procedures described by Wang and Messing (2002) with slight modifications: control without parasitoids (T7), simultaneous exposure in different proportions (T6) and (T3), were added to the normal single, simultaneous and sequential release, for feeding and stinging assessment. The two parasitoids were exposed to 2nd – 3rd-instar larvae of *L. huidobrensis* in six different treatments: (T1) *P. scabriventris* alone (50 adults in the sex ratio of 1: 2, males: females) for 24 hours, (T2) *D. isaea* alone (50 adults in the sex ratio of 1: 2, males: females) for 24 hours; (T3) 50 adult *P. scabriventris* (in the sex ratio of 1: 2, males: females) and 50 adult *D. isaea* (in the sex ratio of 1: 2, males: females) simultaneously for 24 hours, (T4) 50 adult *P. scabriventris* (in the sex ratio of 1: 2, males: females) first for 24 hours, followed by introduction of 50 adult *D. isaea* (in the sex ratio of 1: 2, males: females) for another 24 hours, (T5) 50 adult *D. isaea* (in the sex ratio of 1: 2, males: females) first for 24 hours, followed by 50 adult *P. scabriventris* (in the sex ratio of 1: 2, males: females) for another 24 hours, (T6) 25 adult *P. scabriventris* (in the sex ratio of 1: 2, males: females) and 25 adult *D. isaea* (in the sex ratio of 1:2, males: females) simultaneously for 24 hours and (T7) control, *L. huidobrensis* alone without parasitoids for 24 hours. All the exposed parasitoids were removed from the cages 24 hours post-exposure using a mouth aspirator and test-plants were maintained for larval development for 5 - 7days post-exposure to *L. huidobrensis*.

Interspecific effects on pupal production and adult emergence

To assess the effects of parasitoid interactions on their ability to suppress host population, the numbers of emerged *L. huidobrensis* adults and parasitoids were recorded, sexed, and parasitism rates were also calculated. Pupae were collected 3 - 5 days post-exposure to parasitoids, counted and transferred individually into small transparent plastic capsules (0.70 cm diameter x 2.20 cm high) and incubated at 27 ± 2 ºC, 12L: 12D and 40 – 60 rh until fly or parasitoid emergence.

Host feeding and stinging assessment

To assess effects of parasitoid host-larval feeding and stinging (larval and pupal mortalities), *L. huidobrensis* was maintained alone (without parasitoids) for 24 hours as control (treatment T7) and a number of pupae and adults obtained were used for *L. huidobrensis*'s natural mortality correction in the other six treatments using Abbott's methodology (Abbott, 1925) as compared to parasitoid induced mortality. After emergences, pupae with no exit holes were dissected under a binocular microscope to record the relative mortality of *L. huidobrensis*, *P. scabriventris* and *D. isaea* species. The *L. huidobrensis* pupal mortality was calculated as the percentage of empty pupae and pupae in which a host stage of *L. huidobrensis* was found during the dissection divided by the total number of pupae formed. The relative parasitoid mortality was estimated as the percentage of pupae in which a host stage of a parasitoid was found divided by the total number of pupae formed. Pupae from which no flies or parasitoids were found (empty pupae) were also recorded during the dissection. Differences in relative pupal mortality (non-emerged and empty pupae) and larval mortality (larvae which failed to pupate) using Abbott's methodology were used to determine the feeding-stinging effects of parasitoids.

Treatments were randomized in complete block design (RCBD) and the experiment replicated five times.

4.1.4 Statistical analyses

Parasitism rates, mortality, number of pupae, leafminer fly and parasitoid emergence data were analyzed using Analysis of Variance (ANOVA) in General Linear Model (GLM) procedure in R (2.13.1) statistical software packages (R Development Core Team, 2011). The number of pupae was Log-transformed [Log_{10} (x + 1)] before ANOVA analysis while the emergence rate and pupal mortality percentages were Square Root transformed [$\sqrt{(x + 1)}$]. Tukey HSD multiple comparisons of means were used to separate the means at the significance level of 0.05.

Pupae collected in each treatment were recorded and data were corrected with control using Abbott's formula (Abbott, 1925) to obtain larval and pupal mortalities due to feeding and stinging.

The success rate (%) of parasitism was calculated as follows:

$$\% \text{ parasitism} = \frac{\text{Number of emerged parasitoid species}}{\text{Total number of emerged parasitoids and flies}} \times 100$$

Chi-square tests were used to analyze the comparisons between the various treatments for the parasitoid interaction effects R (2.13.1).

4.2 Results

4.2.1 Effects of interactions between *P. scabriventris* and *D. isaea* on parasitism of *L. huidobrensis*

Data on parasitism are presented in Table 4.1. Specific parasitism was significantly higher with *P. scabriventris* than with *D. isaea*'s whether exposed alone or combined ($p < 0.001$), except in T3 ($p = 0.7784$) (Table 4.1). Specific parasitism by *D. isaea* was not significantly different ($F = 1.91$, $df = 5, 24$, $p = 0.129$) whether used alone or combined with *P. scabriventris* (Table 4.1). The sequence of release of parasitoids did not have any significant effect on the parasitism rate of *D. isaea* (Table 4.1). The specific parasitism of *P. scabriventris* was significantly higher ($F = 14.77$, $df = 5, 24$, $p < 0.0001$) when used alone (T1) compared to when combined with *D. isaea* (T3 – T5) (Table 4.1). The sequence of introduction of *P. scabriventris*, either simultaneously with *D. isaea* (T3), and sequential: before (T4) or after *D. isaea* (T5) did not affect the parasitism by *P. scabriventris*.

Table 4.1: Interactions between *Phaedrotoma scabriventris* (Ps) and *Diglyphus isaea* (Di) and their respective parasitism on *Liriomyza huidobrensis* larvae in *Vicia faba* plants

		Mean % parasitism (X ± SE) of each parasitoid in the total parasitism				
Treatments	**Total % parasitism**	***Phaedrotoma scabriventris***	***Diglyphus isaea***	**Specific and combined parasitism comparison**	**Chi-square (X^2)**	**P-value**
T1 (Ps50)	63.6 ± 7.7 [b]	63.6 ± 7.7[b]	–	**T1 vs. T2**	87.9	< 0.0001
T2 (Di50)	30.4 ± 10.9 [a]	–	30.4 ± 10.9 [a]			
T3 (Ps50 + Di50)	77.0 ± 5.3 [c]	44.7 ± 7.1 [a]	32.3 ± 12.0 [a]	**T3Ps vs. T3Di**	0.10	= 0.7784
T4 (Ps50 → Di50)	63.6 ± 3.3 [b]	47.5 ± 3.8 [a]	16.2 ± 4.5 [a]	**T4Ps vs. T4Di**	39.1	< 0.0001
T5 (Di50 → Ps50)	52.0 ± 9.1 [ab]	31.2 ± 4.9 [a]	20.8 ± 7.8 [a]	**T5Ps vs. T5Di**	9.3	< 0.0022
T6 (Ps25 + Di25)	58.2 ± 5.1 [ab]	37.2 ± 6.1 [a]	21.0 ± 8.9 [a]	**T6Ps vs. T6Di**	10.7	< 0.0011

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

Key: T1 = 50 *P. scabriventris* alone exposed for 24h; **T2** 50 *D. isaea* alone exposed for 24h; **T3** = 50 *P. scabriventris* and 50 *D. isaea* exposed together for 24h; **T4** = 50 *P. scabriventris* exposed for the first 24h and followed by 50 *D. isaea* exposed for another 24h; **T5** = 50 *D. isaea* exposed for the first 24h followed by 50 *P. scabriventris* exposed for another 24h and **T6** = 25 *P. scabriventris* and 25 *D. isaea* exposed together for 24h.

A higher parasitism rate was obtained when 50 of each parasitoid was introduced simultaneously (T3) than when using each parasitoid separately or combining them sequentially. There were no significant differences between the two sequential exposures (T4 and T5) (Table 4.1). There was a significant additive effect through the combination of both parasitoids. While using 50 parasitoids in 24 hours under the same conditions, total parasitism using *D. isaea* alone was significantly lower than a combination in the same ratio with *P. scabriventris* (T6) ($X^2 = 27.7$, $df = 1$, $P_{(T2\ vs.\ T6)} < 0.0001$), while no significant differences were found between *P. scabriventris* alone (T1) and mixtures of same ratio (T6) ($X^2 = 18.3$, $df = 1$, $P_{(T1\ vs.\ T6)} < 0.0001$). The number of parasitoids released (50 or 100 each), (T3) and (T6), influenced the total rates of parasitism, with higher numbers of parasitoids leading to higher total parasitism rate (Table 4.1).

4.2.2 Feeding and stinging-induced mortality

The larval “feeding-stinging mortality” (host-feeding and stinging) of *D. isaea* was 41.9% when exposed alone (Table 4.2). Although the “emergence parasitism” of *D. isaea* was only 30.4% ± 10.9, the total mortality of *L. huidobrensis* larvae caused by *D. isaea* when considering the “feeding-stinging parasitism” was 62.1%. When exposed together (T3) and taking into account “feeding-stinging mortality”, the total mortality reached 91.5% compared to 77.0% obtained without larval mortality caused by *D. isaea* host-feeding and stinging. The feeding-stinging phenomenon was also observed in the endoparasitoid *P. scabriventris* but at lower rate as compared to *D. isaea*. The larval mortality due to host-feeding-stinging for *P. scabriventris* when exposed alone was 11.9 ± 8.7% and when exposed together with *D. isaea* (T5), it increased to 44.7 ± 6.0% (Table 4.2).

Table 4.2: Larval and pupal mortalities due to feeding and stinging by *Diglyphus isaea* and *Phaedrotoma scrbriventris*

	Mean % mortalities (X ± SE) due to feeding and stinging	
Treatments	**Larval**	**Pupal**
T1 (Ps50)	11.9 ± 8.7[a]	21.6 ± 1.9[a]
T2 (Di50)	41.9 ± 9.1[b]	49.1 ± 6.5[b]
T3 (Ps50 + Di50)	44.7 ± 6.01[b]	40.7 ± 5.4[ab]
T4 (Ps → Di)	24.5 ± 4.7[ab]	44.3 ± 6.4[ab]
T5 (Di50 → Ps50)	32.9 ± 5.2[ab]	47.1 ± 5.8[b]
T6 (Ps25 + Di25)	34.7 ± 2.8[ab]	39.2 ± 5.2[ab]

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

Key: T1 = 50 *P. scabriventris* alone exposed for 24h; **T2** 50 *D. isaea* alone exposed for 24h; **T3** = 50 *P. scabriventris* and 50 *D. isaea* exposed together for 24h; **T4** = 50 *P. scabriventris* exposed for the first 24h and followed by 50 *D. isaea* exposed for another 24h; **T5** = 50 *D. isaea* exposed for the first 24h followed by 50 *P. scabriventris* exposed for another 24h and **T6** = 25 *P. scabriventris* and 25 *D. isaea* exposed together for 24h.

Similarly, pupal mortality due to feeding-stinging when *D. isaea* was exposed alone was 49.1 ± 6.5% as compared to 21.6 ± 1.9% with the endoparasitoid *P. scabriventris* (Table 4.2). Additionally, the dissection showed that 30.8 ± 1.8% of the pupae were empty (with dried content) in T2 (*D. isaea* alone) and only 18.2 ± 3.8% in T1 (*P. scabriventris* alone) as compared to 5.9 ± 0.9% in the control T7 (Table 4.3).

Table 4.3: Mean pupal mortality percentage of *Liriomyza huidobrensis*, *Phaedrotoma scabriventris* and *Diglyphus isaea* following dissection of non-emerged pupae

	Mean % pupal mortality (X ± SE) of species after dissection			
Treatments	***Liriomyza huidobrensis***	***Phaedrotoma scabriventris***	***Diglyphus isaea***	**Number of Empty pupae cases**
T1 (Ps50)	75.9 ± 4.3[a]	5.8 ± 0.9[ab]	–	18.2 ± 3.8[b]
T2 (Di50)	67.7 ± 1.9[ab]	–	1.6 ± 0.6[a]	30.8 ± 1.8[a]
T3 (Ps50 + Di50)	64.7 ± 1.9[b]	10.2 ± 1.5[c]	1.0 ± 0.5[a]	24.2 ± 1.8[ab]
T4 (Ps50 → Di50)	73.4 ± 1.2[ab]	6.8 ± 1.6[b]	0.8 ± 0.4[a]	18.9 ± 1.6[b]
T5 (Di50 → Ps50)	72.7 ± 2.9[ab]	4.9 ± 1.4[a]	0.1 ± 0.1[a]	22.3 ± 2.1[ab]
T6 (Ps25 + Di25)	73.3 ± 2.6[ab]	5.7 ± 1.7[ab]	0.9 ± 0.4[a]	20.1 ± 0.8[b]
T7 Control (Lh)	94.1 ± 0.9[c]	–	–	5.9 ± 0.9[c]

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

Key: T1 = 50 *P. scabriventris* alone exposed for 24h; **T2** 50 *D. isaea* alone exposed for 24h; **T3** = 50 *P. scabriventris* and 50 *D. isaea* exposed together for 24h; **T4** = 50 *P. scabriventris* exposed for the first 24h and followed by 50 *D. isaea* exposed for another 24h; **T5** = 50 *D. isaea* exposed for the first 24h followed by 50 *P. scabriventris* exposed for another 24h and **T6** = 25 *P. scabriventris* and 25 *D. isaea* exposed together for 24h.

There were significant differences among the treatments in terms of empty pupae percentage ($F = 13.53, df = 6, 28, p < 0.0001$), *L. huidobrensis* that died inside the pupae ($F = 14.35, df = 6, 28, p < 0.0001$) and *P. scabriventris* that died inside the pupae ($F = 8.96, df = 6, 28, p < 0.0001$) (Table 4.3). However, there were no significant differences ($F = 2.79, df = 6, 28, p < 0.029$) observed among the treatments (T2 – T6) regarding *D. isaea* that died inside the pupae (Table 4.3). For instance, from the dissected non emerged pupae, 75.9 ± 4.3% and 67.7 ± 1.9% of the pupae contained

immature/adult *L. huidobrensis* for T1 and T2 respectively and only few immature/adult dead parasitoids (5.8 ± 0.9% for T1 and 1.6 ± 0.6% for T2) (Table 4.3). This same observation was made in the other treatments with both parasitoids either simultaneously or sequential released.

4.2.3 Effects of interaction between *P. scabriventris* and *D. isaea* on sex ratio of parasitoids and host in F1 progeny

Despite the fact that there was no significant difference (*p = 0.765*) in the F1 sex ratio in any of the treatments, it was observed that, in the first generation (F1) of all the treatments of *P. scabriventris*, there were always slightly more males emerged compared to females (Table 4.4).

Table 4.4: Effect of interaction between *Phaedrotoma scabriventris* and *Diglyphus isaea* on the F1 progeny sex ratio

	Liriomyza huidobrensis		***Phaedrotoma scabriventris***		***Diglyphus isaea***	
Treatments.	**% Male**	**% Female**	**% Male**	**% Female**	**% Male**	**% Female**
T1 (Ps50)	58.4	41.6	54.9	45.1		
T2 (Di50)	57.3	42.7	–	–	42.7	57.3
T3 (Ps50 + Di50)	60.7	39.3	56.2	43.8	40.9	59.1
T4 (Ps50 → Di50)	58.7	41.4	54.5	45.5	58.9	41.1
T5 (Di50 → Ps50)	56.5	43.5	57.0	42.9	46.9	53.0
T6 (Ps25 + Di25)	57.1	42.9	52.2	47.8	49.5	50.5
T7 Control (Lh)	51.4	48.6	–	–	–	–

Key: T1 = 50 *P. scabriventris* alone exposed for 24h; **T2** 50 *D. isaea* alone exposed for 24h; **T3** = 50 *P. scabriventris* and 50 *D. isaea* exposed together for 24h; **T4** = 50 *P. scabriventris* exposed for the first 24h and followed by 50 *D. isaea* exposed for another 24h; **T5** = 50 *D. isaea* exposed for the first 24h followed by 50 *P. scabriventris* exposed for another 24h and **T6** = 25 *P. scabriventris* and 25 *D. isaea* exposed together for 24h.

However, more females (>50%) than males emerged in *D. isaea* treatments, except in (T4) where *P. scabriventris* was introduced first for 24 hours followed by *D. isaea* with 41.1% ♀ vs. 58.9% ♂ (Table 4.4). There was then no detrimental effect on the sex ratio of the parasitoids when co-existing i.e. whether they were single, simultaneously or sequentially released.

Solitary parasitism

In all cases, only one parasitoid was obtained from each capsulated pupa. From 7402 pupae collected from all the parasitoid treatments and capsulated, none of them gave double emergence. For instance, in the simultaneous (T3 and T6) and sequential (T4, T5) releases, there were never two parasitoids collected from one capsule. Only one parasitoid per capsule was collected. There was no superparasitism/multiparasitism or hyperparasitism since no double emergence was observed in all the treatments. No mutually detrimental effect was observed between *P. scabriventris* and *D. isaea* interaction where in limited resource sharing for progenies generation of the two parasitoids.

4.3 Discussion

The simultaneous use of *P. scabriventris* and *D. isaea* resulted in a higher parasitism rate compared to each of them used separately. The parasitism rate of *D. isaea* and *P. scabriventris* obtained in the present study was similar to the one reported elsewhere. For instance, Boot *et al.* (1992) reported 30% parasitism by *D. isaea* in *L. bryoniae*. *Phaedrotoma scabriventris* performed better than *D. isaea* in terms of specific parasitism, showing that it is a superior parasitoid compared to *D. isaea* and therefore its release in the horticultural production systems will fill the existing gap of lower parasitism rates reported in Kenya by Chabi-Olaye *et al*. (2008) and substantially contribute to the suppression of *Liriomyza* species as well as enhancing horticulture production in Kenya. In addition, *P. scabriventris* did not have any negative effect on the performance of *D. isaea*. Since only a single parasitoid was obtained from each pupa in

all treatments observed, this study confirmed that the two parasitoid species used in the study are both solitary parasitoids. Even in (T4) and (T5) where *P. scabriventris* and *D. isaea* were sequentially introduced, and in (T3) and (T6) where both parasitoids were simultaneously exposed, two parasitoids were never obtained from a capsulated pupa. This also shows that there was no multiparasitism (repeated parasitism of a host by parasitoids of different species), hyperparasitism (specialized as parasitoids of other parasitoids, seeking parasitize hosts and laying eggs into the host or into the developing parasitoid within) or superparasitism (repeated parasitism of a host by parasitoids of the same species) among and between *D. iasea* and *P. scabriventris*. Although *P. scabriventris* had no effect on *D. isaea*, the presence of *D. isaea* reduced slightly the performance of *P. scabriventris* in terms of parasitism. However, both parasitoids used together led to a higher total parasitism. This is an indication that no harmful effect under field conditions can be expected after release of *P. scabriventris* in east Africa and the association of both parasitoids will result into a more pronounced reduction of the leafminer flies in East Africa.

Successful co-existence of *P. scabriventris* and various *Diglyphus* species was reported by Mujica and Kroschel, (2007), Valladares *et al*. (1999) and Kroschel, (2008). According to Minkenberg, (1989) *D. isaea* occurs widely as an ectoparasitoid of leafmining dipteran larvae on herbaceous plants in Europe, Northern Africa and Japan. *Diglyphus isaea* is usually a solitary larval ectoparasitoid of agromyzid leafminers including *L. huidobrensis*, *L. sativae* and *L. trifolii* (Musundire, 2002; Ode and Heinz, 2002; Liu *et al*., 2009; Kang *et al*., 2009). Parasitoid competitions in leafminers are not known and hyperparasitism has never been observed in any leafminer fly field samples taken over many years in Peru and Kenya (Murphy and LaSalle, 1999; Mujica and Cisneros, 1997; Mujica and Kroschel, 2007; Chabi-Olaye *et al*., 2008). For instance, in Peru, the leafminer fly complex has more than 60 different parasitoid species which co-exist (Mujica and Kroschel, 2007). It is therefore expected that, after the release of the introduced parasitoid *P. scabriventris*, higher parasitism rates of leafminer flies will be obtained and hence better biological control achieved.

In addition to the mortality induced by *P. scabriventris* and *D. isaea* through oviposition (parasitism rate) discussed above, another very important additional mortality was caused through parasitoid feeding-stinging behavior and needs to be accounted for when evaluating their performance. Minkenberg (1989) and Anonymous (2007) also reported that the females *D. isaea* not only lay eggs on late second and third instar larvae, but also host feed on larvae (host feeding), sting and introduce toxin into larvae (host stinging) before laying eggs. Host feeding-stinging by parasitoids therefore considerably reduced the offspring of the *L. huidobrensis* population beyond the simple parasitism rate. Many synovigenic parasitoids use their host insects either as oviposition sites or as food sources, a behavior called “host-feeding” (DeBach, 1943; Jervis and Kidd, 1986). By feeding upon host, females can improve their longevity, fecundity, and searching efficiency (Syme, 1977). In the same line of feeding-stinging phenomenon, Uematsu (1986) reported that the ectoparasitoid *Euplectrus kuwanae* Crawford (Hymenoptera: Eulophidae) paralyzed its host temporally by pre-ovipositional stinging to avoid attack from the non-parasitized host at the time of oviposition. Mafi and Ohbayashi (2010) also reported that feeding and stinging without oviposition of *Sympiesis striatipes* Ashmead (Hymenoptera: Eulophidae), an ectoparasitoid of the citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), killed 44.7 ± 4.2 host larvae per female parasitoid, of which around 60% was caused by host feeding or stinging activity of adult females. In addition to the parasitism rates of 20 to 30% reported for *D. isaea* in the current study, the mortalities induced by this ectoparasitoid to larva and pupa *L. huidobrensis* through feeding-stinging were around 41.9 and 49.1% respectively. The mortalities induced through feeding and stinging activities of the endoparasitoid *P. scabriventris* were 5.6 times lower than that recorded for *D. isaea*. However, the presence of *P. scabriventris* did not affect significantly the feeding-stinging mortalities induced by *D. isaea*.

4.4 Conclusion

Phaedrotoma scabriventris performed better than *D. isaea* in terms of parasitism rate and represent a superior parasitoid compared to *D. isaea.* Its importation and release will fill the existing gap of lower parasitism rate reported in Kenya. On the other hand, *D. isaea* cause a considerably higher feeding and stinging mortality to *L. huidobrensis* than found in *P. scabriventris*. Additionally, *P. scabriventris* has no negative effect on the parasitism rate or feeding and stinging mortalities caused by *D. isaea.* Simultaneous use of both parasitoids led to higher total parasitism and mortalities due to feeding and stinging. No multiparasitism, superparasitism or hyperparasitism were observed within and among the two parasitoids. This shows that no harmful effect is expected on *D. isaea* after release of *P. scabriventris* in East Africa and the association of both parasitoids will result into a more pronounced mortality and parasitism of the leafminer flies in East Africa, hence a better biological control of the pest.

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CHAPTER 5: MORPHOLOGICAL AND MOLECULAR CHARACTERIZATIONS OF FUNGAL ENDOPHYTES ISOLATED FROM *VICIA FABA* AND *PHASEOLUS VULGARIS* DRIED SEEDS (FABACEAE)

Abstract

Fungal endophytes are heterotrophic microorganisms occurring inside plant tissues and have been detected in hundreds of plants. Some of them have been shown to have adverse effects against insects, nematodes, and plant pathogens. An initiative is underway at the International Centre of Insect Physiology to use them as a novel strategy for the control of *Liriomyza* leafminer. It is in this context that bioprospecting for isolation of fungal endophytes in the seeds of *Phaseolus vulgaris* and *Vicia faba* was undertaken. Isolated fungi were identified using morphological and molecular techniques; they included *Beauveria bassiana*, *Epacris microphylla*, *Phanerochaete chrysosporium* and *Metarhizium anisopliae*. The identities of all the endophytes were between 99 and 100% with an E-value of 0.0.

5.0 Introduction

The term endophyte was coined by the German scientist Heinrich Anton De Bary (1884), and is used to define fungi or bacteria occurring inside plant tissues without causing any apparent symptoms in the host (Wilson, 1995). They are heterotrophic microorganisms (Ingram, 2002) that live inside plants primarily for nutrition, protection and reproduction. Some of them are beneficial while others may be pathogenic to crops (Azevedo *et al*., 2000; Backman and Sikora, 2008). Fungal endophytes have been detected in hundreds of plants, including many important agricultural commodities such as wheat (Larran *et al*., 2002a), bananas (Pocasangre *et al*., 2000; Cao *et al*., 2002), soybeans (Larran *et al*., 2002b), and tomatoes (Larran *et al*., 2001). *Beauveria bassiana* (Balsamo) Vuillemin has been reported as an endophyte in maize (Bing and Lewis, 1991, 1992a, b; Lomer *et al*., 1997; Cherry *et al*., 2004), potato, cotton, cocklebur, jimson weed (Jones, 1994) tomato (Leckie, 2002; Ownley *et al*., 2004), in seeds and

needles of *Pinus monticola* Dougl. ex. D. Don (Ganley and Newcombe, 2005), in opium poppy (Quesada-Moraga *et al*., 2006), in bananas (Akello *et al*., 2007), and on several other crops (Posada *et al*., 2007; Go´mez-Vidal *et al*., 2006).

It is likely that every plant species harbours endophytes, and indeed seeds of many plant species have been reported to harbour endophytes (Mundt and Hinkle, 1976; Schardl *et al*., 2004). Plant seeds usually fall into the soil, a microbially rich habitat, and lie dormant waiting for environmental cues to germinate, possibly recruiting surface microbes to help protect them against degradation or predation (Dalling *et al*., 2011). As seeds begin to germinate, seed endophytes may be important founders of the seedling microbial community as shown in rice (Kaga, *et al*., 2009; Mano *et al*., 2006), eucalyptus (Ferreira *et al*., 2008) and maize (Rijavec *et al*., 2007). Seeds are of particular interest as they may transmit endophytes vertically from generation to generation (Johnston-Monje and Raizada, 2011 b).

Several roles have been ascribed to fungal endophytes, including providing protection against herbivorous insects (Breen, 1994; Clement *et al*., 1994), plant parasitic nematodes (West *et al*., 1988; Elmi *et al*., 2000), and plant pathogens (Dingle and McGee, 2003; Wicklow *et al*., 2005).

Liriomyza spp. (Diptera: Agromyzidae) leafminers are invasive pests of horticultural crops which originated from the New World (Murphy and LaSalle, 1999). The main management strategy focuses on the use of synthetic chemical insecticides which do not provide efficient control against the pest due to the development of resistance and elimination of natural enemies, in addition to health risks and pesticide residues. At the International Centre of Insect Physiology (*icipe*), biological control through the use of parasitoids and entomopathogens is being developed as an integrated management strategy for leafminer control. Among the entomopathogens, the use of fungal endophytes is being considered. Bioprospecting was undertaken for isolation of fungal endophytes in *Phaseolus vulgaris* and *Vicia faba* seeds. Morphological and molecular

tools were used to identify and characterize these fungal endophytes isolated from the two legume seeds.

5.1 Materials and Methods

5.1.1 Seeds

Phaseolus vulgaris (Rose coco beans) and *Vicia faba* (Faba beans) seeds which are local Kenyan Open Pollinated varieties were collected from different sources, especially from local markets (Nairobi, Kasarani, Eastleigh, Nyamakima, Gikomba and sometimes from Ethiopia to Kenya's markets) and some super markets (Naivas supermarket, since farmers use these seeds for sowing).

5.1.2 Fungal colonization

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 (Schulz and Boyle, 2005). Three to five seeds were plated onto 9-cm Petri dishes containing PDA, SDA and Yeast Extract Agar (YEA).

To determine the growth/colonization of fungal endophytes in *V. faba* and *P. vulgaris*, the different seeds, grinded or not (whole), were placed on PDA, SDA and YEA plates (distant of 4 cm each) amended with a 0.05% of antibiotic (Streptomycin sulfate salt and Chloramphenicol) (Dingle and McGee, 2003; Istifadah and McGee, 2006; Gurulingappa *et al.*, 2010). Plates were incubated at 25 ± 1 ^{o}C for 14 days, after which the presence of endophytes was determined. Prior to incubation of the different sterilized seeds, the last rinse water was also plated out to assess the effectiveness of the surface sterilization procedure (Schulz and Boyle, 2005). The colonization of the different seeds was recorded by counting the number of seeds of the different varieties that showed fungal growth/mycelia according to Koch's postulates (Petrini and Fisher, 1987). This experiment was repeated five times.

5.1.3 Fungal isolation and subcultures

After confirming Koch's postulates, isolated fungi were sub-cultured for 4 – 6 times to obtain pure cultures for morphological and molecular identification.

5.1.4 Morphological identification

For morphological identification, fungal colony features such as appearance, texture (colonization pattern) and pigmentation on both the top and reverse plates were observed for each isolate and then used to group isolates into morphospecies (Kornerup and Wancher, 1978; Burgess *et al*., 1994; Humber, 1997; Leslie and Summerell, 2006). After that, micro-morphological characteristics of each isolate were observed under the light microscope. For this, the spores or/and mycelia of each isolate were harvested and then stained with Lacto Phenol Cotton Blue for 10 to 15 min. A drop of the resultant suspension was placed on a slide, covered with a cover slip and then examined with the x40 objective. Some of the characteristics used in the identification and classification of endophytic fungal species encompassed: i) presence or absence of microconidia and macroconidia, ii) shapes and sizes of conidia, iii) type of phialides bearing microconidia, iv) presence or absence of chlamydospores, microconidial chains and sporodochia, and v) type of fungal mycelia (Akello, 2012).

5.1.5 Molecular characterization

5.1.5.1 Preparation of fungal material

Conidia were harvested by scraping the surface of 1 to 2-week old cultures with a sterile spatula. The harvested conidia/mycelia were then mixed with the buffer for Fast DNA Extraction process. *Beauveria bassiana* isolate ICIPE279 and *Metarhizium anisopliae* isolate ICIPE30 and S4ST7 which were isolated from a Coleopteran larva, *Busseola fusca* pupa and aboveground parts of maize, sorghum and Napier grass, respectively, were added as standards. They were obtained from the *icipe*'s Arthropod Germplasm Centre. The sub-cultures of the isolated fungi were re-isolated and again sub-cultured for 4 – 6 times before using them for morphological and molecular studies.

Mycelia/conidia plates of 1 to 2 weeks old were freeze-dried for 24 hours to facilitate easy grinding. The dried conidia/mycelia were harvested by scraping the surface with a sterile spatula. Five hundred milligrams of fungal conidia/mycelia were then mixed with the extraction buffer inside an Eppendorf tube containing beads, followed by vortexing for 2-5 min. for DNA extraction process.

5.1.5.2 Genomic DNA extraction

Genomic DNA was extracted using Fast DNA Spin® Kit for Soil, where 500 mg of conidia/mycelia was added to Lysing Matrix E Tube. After that 978 µl of Sodium Phosphate Buffer and 122 µl of MT Buffer were added. Tubes were secured in FastPrep® Instrument and processed for 30 seconds at speed 5.5. The Lysing Matrix E Tubes were centrifuged at 14,000 xg for 30 seconds followed by the transfer of the supernatant to clean tubes. Two hundred and fifty (250) µl of PPS reagent was added and mixed by shaking the tube by hand 10 times. It was then centrifuged at 14,000 xg for 5 minutes to precipitate the pellet. The supernatant was transferred into a clean 1.5 ml tube (Re-suspend Binding Matrix Suspension before use) and 1 ml of Binding Matrix Suspension was added to the supernatant. The tubes were placed on rotator or invert by hand for 2 minutes to allow binding of DNA to matrix after which they were placed in a rack for 3 minutes to allow settling of silica matrix. Five hundred (500) µl of the supernatant was carefully removed to avoid unsettling the Binding Matrix and the supernatant discarded. The Binding Matrix was then re-suspended in the remaining amount of supernatant, after which approximately 600 µl of the mixture was transferred to a SPIN^{TM} Filter and centrifuged at 14,000 xg for 1minute. The Catch tube was emptied and the remaining supernatant was added to the SPIN^{TM} Filter and spun again. Five hundred (500) µl of SEWS-M was added to SPIN^{TM} Filter and centrifuged at 14,000 xg for 1 minute, after which the flow-through was decanted and the SPIN^{TM} Filter was replaced in the catch tube followed by centrifugation at 14,000 xg for 2 minutes to “dry” the matrix of residual SEWS-M wash solution. The SPIN^{TM} Filter was removed and placed in a fresh catch tube for air drying for 5 minutes at room temperature. After all this, 50 µl of DES (Dnase/Pyrogen Free Water) was added and the matrix was gently

stirred on filter membrane with a pipette tip or votex/finger flip to re-suspend the silica for efficient elution of the DNA. This was followed by centrifugation at 14,000 xg for 1 minute to transfer eluted DNA to clean catch tubes. The DNA was stored at -20 ºC until required for PCR.

5.1.5.3 Amplification and Polymerase Chain Reaction (PCR) using ITS 5 & 4 and AB28 & TW81 primers

The isolated DNA was amplified in 30 µl PCR mix. Amplifications were carried out for the rDNA region of the fungal isolates using the ITS 5 & 4 and AB28 & TW81 primers (White *et al*., 1990). For identification of the fungi, not only a molecular method of moderate resolution (ITS regions) was used identifying a genus and species of fungus, but also combined with AB28 & TW81 regions for a good measure of the variability within an isolate species. The reaction mixture consisted of 200 µM of each dNTP (dATP, dTTP, dCTP and dGTP), 1.5 units of GoTaq® Green reaction buffer (Genscript buffer), 18.6 units of H_2O, 0.2 µM of each primer set (ITS 5 (Forward) = 5' GGAAGTAAAAGTCGTAACAAGG 3' and ITS 4 (Reverse) = 5' TCCTCCGCTTATTGATATGC 3' and TW81 (Forward) = 5' GTT TCC GTA GGT GAA CCT GC 3' and AB28 (Reverse) = 5' ATA TGC TTA AGT TCA GCG GGT 3') respectively, and 3 µl of genomic DNA.

The amplification was conducted using two different programs. The cycling program for the ITS region amplification was initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 40 sec, annealing at 58 °C for 40 sec, and primer elongation at 72 °C for 1 min followed by a final extension at 72 °C for 10 min giving a product range of between 550 - 600 bp. The same program was applied for AB28 & TW81 primers, except that the denaturation at 94 °C for 40 sec was set for 40 cycles and the annealing temperature at 57 °C for 40 sec. This gave a target region of ranging between 500 - 550 bp. Both reactions were done in a PTC 100 thermocycler (MJ Research, Gaithersburg).

5.1.5.4 DNA purification and sequencing

Agarose gel electrophoresis

For agarose gel electrophoresis, 1% agarose gel was prepared by dissolving 1 mg of agarose powder into 100 ml of 1 X TAE buffer, then the mixture stirred and boiled in a microwave. Five microlitre of ethidium bromide (10 µg/ml) was added and then the mix was allowed to cool before being poured into the casting trays for polymerization. Seven (7) µl of the PCR product was mixed with the loading dye and samples loaded into the gel, alongside 100 bp molecular weight DNA ladder. Electrophoresis was set at 70 volts for 1 hr (Bio-Rad model 200/2-0 power supply and wide mini-sub cell GT horizontal electrophoresis system, Bio-Rad laboratories, Inc., USA), followed by visualization of the DNA under UV-illumination. The gel was photographed analyzed and documented using KODAK Gel Logic 200 Imaging System software (Raytest, GmbH**,** Straubenhardt). This was done using the QuickClean 5M Gel Extraction Kit II from GenScript (GenScript Corporation, Piscataway, NJ), following the manufacturer's instructions and subsequently sequenced in both directions using ABI 3700 genetic analyzers.

Sequencing and molecular data analysis

The purified PCR products were quantified using the Eppendorf Biophotometer and sent to Macrogen Inc in South Korea for sequencing. The sequences obtained were assembled and edited using Chromas version 2.13 (Technelysium Pty ltd, Queensland, Australia) consensus sequences from both the forward and reverse strands were generated (Annexes 2 and 3). The consensus reads generated were then queried through BLASTN, in the GenBank database provided by the National Centre of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) for identification purposes and to check for similarity with organisms already identified. Any isolate exhibiting ≥ 95% sequence similarity to NCBI strains was considered as the correct species for that isolate (Arnold and Lutzoni, 2007).

Moreover, the cleaned sequences were aligned using ClustalX version 1.81 (Thompson *et al*., 1997). These alignments were used for phylogenetic and molecular evolutionary analyses that were conducted using MEGA version 5 (Tamura *et al.*, 2011). Neighbour-

joining trees were constructed (Saitou and Nei, 1987) with bootstrapping and using the Kimura 2 distance matrix (Kimura, 1980) for both sets of sequences from the two gene regions. Tables of between species distances were also constructed using MEGA version 5 (Tamura *et al.*, 2011). The tables of distances were used to generate the principal component plots using the program GenAlEx 6.41 (Peakall and Smouse, 2006).

Morphological data analysis

Fungal colonization percentages were Square Root transformed [$\sqrt{(x + 1)}$] before applying Analysis of Variance (ANOVA) in R (2.13.1. Tukey HSD multiple comparisons of means were used to separate the means. The success rate (%) of fungal colonization of seeds was calculated as follows:

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

All the analyses were performed using R (2.13.1) statistical software packages (R Development Core Team, 2011) while relying heavily on the epicalc package (Chongsuvivatwong, 2012).

5.2 Results

5.2.1 Fungal colonization and endophytes diversity using morphological characterization

Irrespective of the seed species, 100% of the sampled seeds were colonized by at least one fungal isolate. The colonization frequency measured as the proportion of seeds yielding isolates ranged from 25 – 100% (Figures 5.1 and 5.3). Using morphological features (texture, pigmentation, spores or/and mycelia, microconidia, macroconidia, shapes and sizes of conidia, type of phialides, microconidial chains and sporodochia, type of fungal mycelia and presence or absence of chlamydospores), five fungal

isolates were grouped into four genera: *Metarhizium* (MF, MR and CR), *Beauveria* (BF), *Epacris* (ZRA) and *Phanerochaete* (ZF) (Figure 5.1).

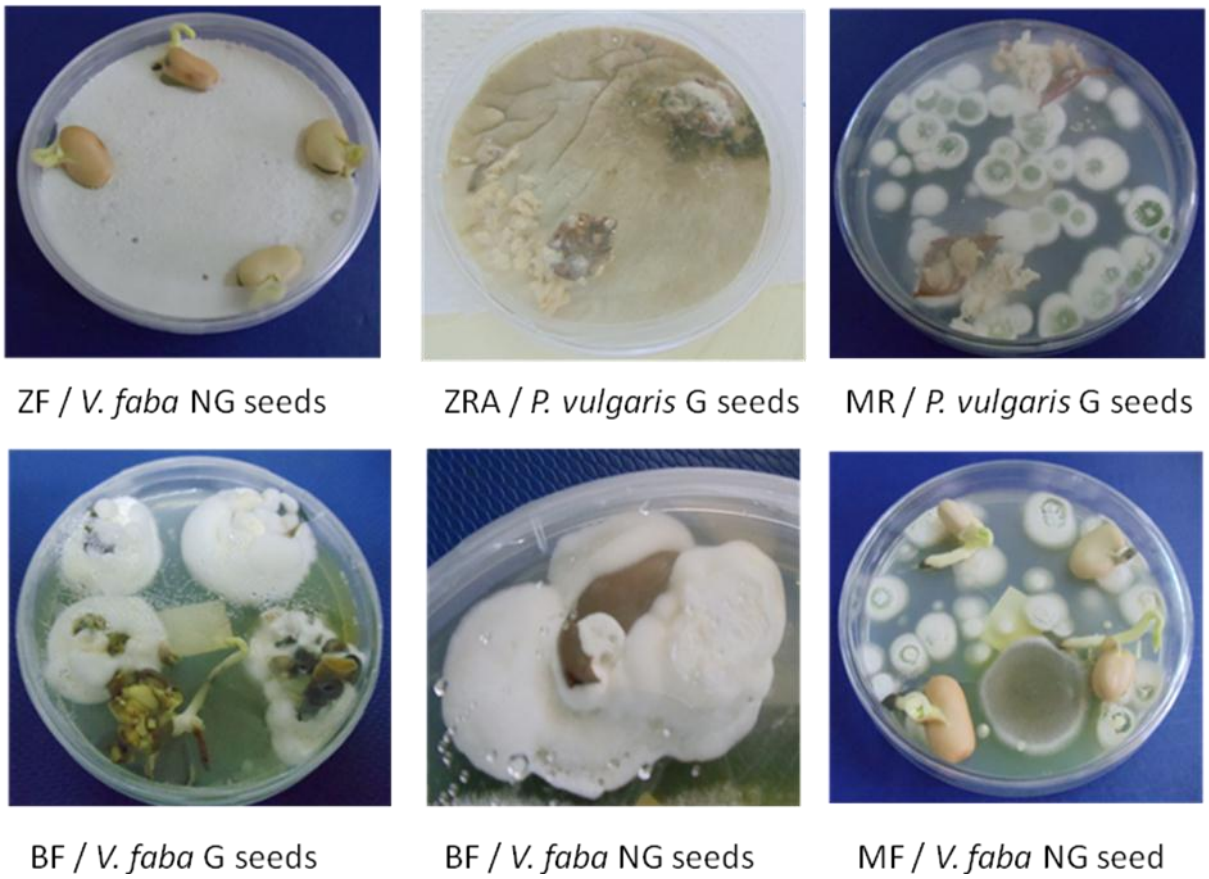

Figure 5.1: Fungal species isolated from *Phaseolus vulgaris* and *Vicia faba* seeds two weeks after incubation. ZF = *Phanerochaete chrysosporium*; ZRA = *Epacris microphylla*; MR = MF = *Metarhizium anisopliae* and BF = *Beauveria bassiana*. G = Grinded seeds; NG = Non grinded seeds.

In addition to the five different species isolated and identified from the seeds, another species of *Metarhizium* (CR) was also isolated from *P. vulgaris* seedling root using molecular tools (Figure 5.2). There was no fungal growth in any of the incubated washing water plates.

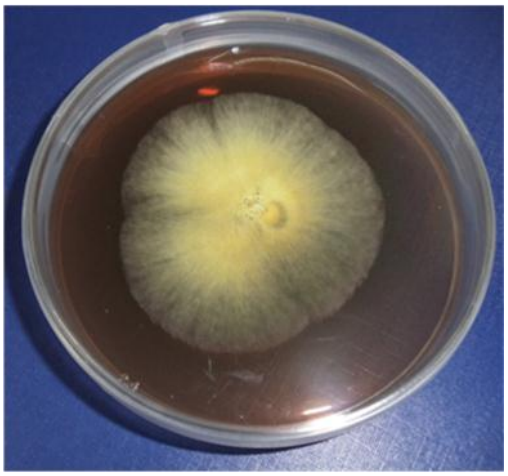

Figure 5.2: *Metarhizium anisopliae* (CR) isolated from *Phaseolus vulgaris* root two weeks after incubation

The colonization pattern of the various endophyte species was also determined (Figure 5.3). There were no significant differences in percentage colonization among *V. faba* seeds ($F(3, 20) = 973.96; df = 3, p < 0.502$) and *P. vulgaris* seeds ($F(3, 20) = 729.91; df = 3, p < 0.707$) (Figure 5.3). For instance *Beauveria bassiana* (BF) colonized 100% of grinded and non grinded seeds of *V. faba* but was not observed in *P. vulgaris* seeds (Figure 5.1). ZF was only observed in *V. faba* with 66.67% colonization whereas ZRA was observed with 55% colonization in only *P. vulgaris*. However, *Metarhizium anisopliae* was found in *P. vulgaris* seeds (55.36%) as well as in *V. faba* seeds (70.83%) (Figure 5.3).

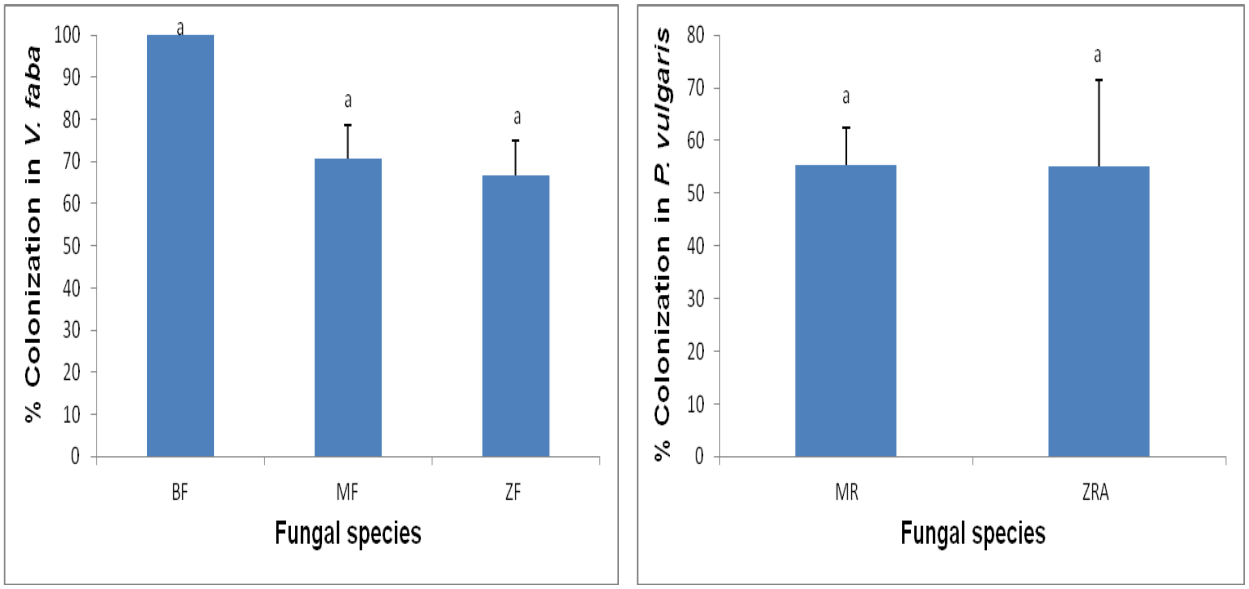

Figure 5.3: Fungal endophyte species % colonization in *Vicia faba* (left) and in *Phaseolus vulgaris* (right) seeds. ZF = *Phanerochaete chrysosporium*; ZRA = *Epacris microphylla*; MR = MF = *Metarhizium anisopliae* and BF = *Beauveria bassiana*.

5.2.2 Fungal endophytes identification and diversity using molecular characterization

PCR Products after Genomic DNA extraction

Below are gel photographs (Figures 5.4 and 5.5) showing PCR products after amplification with the two primer sets, the ITS 5 & 4 and AB28 & TW81 primers.

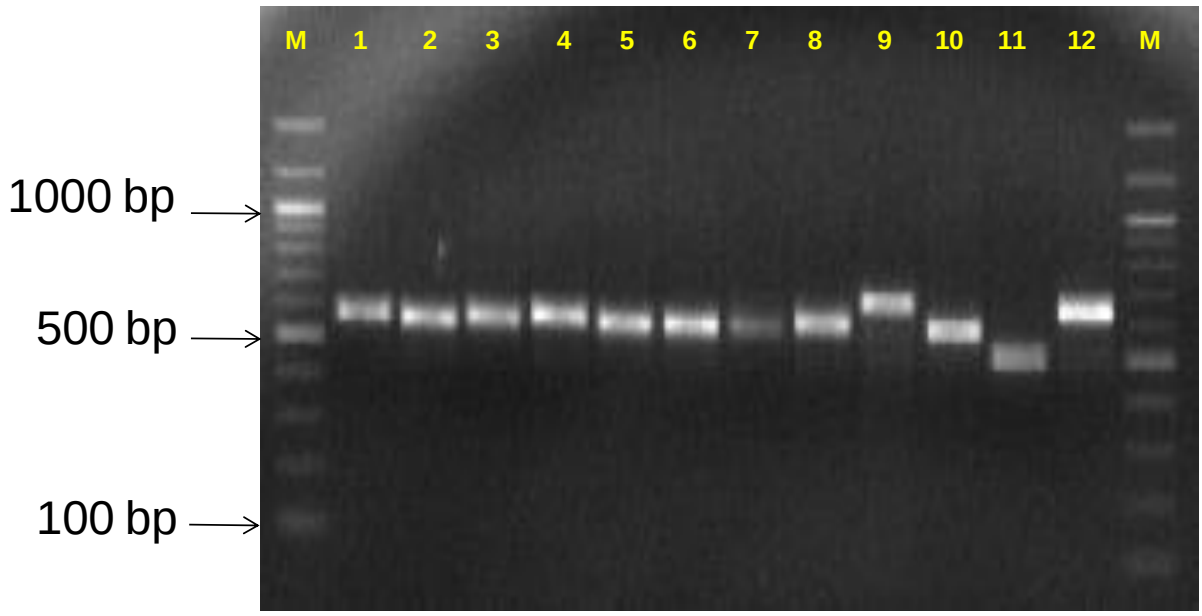

Figure 5.4: PCR products using ITS 4and 5 primers. M: Ladder; 100 ladder (New England Biolabs), Lane 1: ZRA, Lane 2: ZRB, Lane 3: S4ST7, Lane 4: ICIPE 279, Lane 5: MF, Lane 6: MR, Lane 7: CR, Lane 8: BF, Lane 9: ZF, Lane 10: ICEPE 30, Lane 11: Y and Lane 12: X.

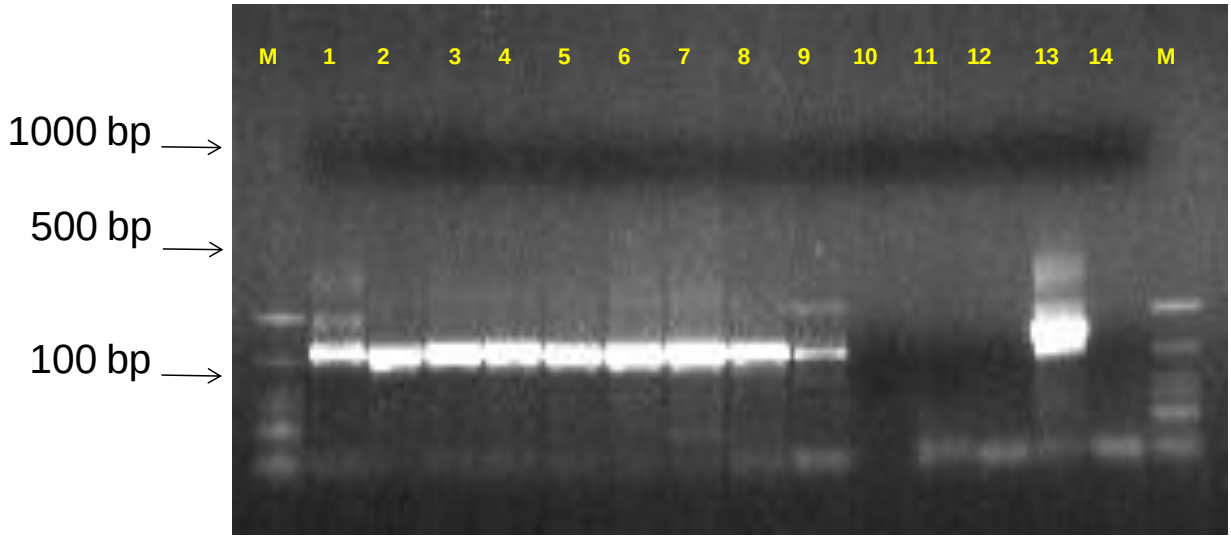

Figure 5.5: PCR products using AB28/TW81 primers. M: Ladder; 100 ladder (New England Biolabs, Lane 1: ZRA, Lane 2: ZRB, Lane 3: S4ST7, Lane 4: ICIPE 279, Lane 5: MF, Lane 6: MR, Lane 7: BF, Lane 8: BFB, Lane 9: CR, Lane 10: CR New, Lane 11: CR Old, Lane 12: CRB, Lane 13: ZF and Lane 14: Negative control.

The provisional accession numbers of these endophyte isolates deposited into the *icipe* germplasm centre are: ZRA = ICIPE 695 (*Epacris microphylla*); ZF = ICIPE 692 (*Phanerochaete chrysosporium*); BF = ICIPE 693 (*Beauveria bassiana*); CR = ICIPE 691 (*Metarhizium anisopliae*); MF = ICIPE 694 (*Metarhizium* anisopliae); MR = ICIPE 696 (*Metarhizium anisopliae*). S4ST7, MF, MR and ICIPE 30 are all *Metarhizium anisopliae* while ICIPE 279 and BF are *Beauveria bassiana* isolates, respectively.

Since ICIPE 30, ICIPE 279 and S4ST7 were used as standards during the identification process, they were therefore considered as references in the phylogenetic trees for sibling or related isolates species characterization.

Fungal isolate identities

The molecular identification of the endophyte samples after sequencing is consolidated in Table 5.1. All the nine fungal isolates identified using ITS 5 & 4 primer set were confirmed by AB28 & TW81 primer set. They all showed the same identity whether using ITS 5 & 4 or AB28 & TW81 with 99 – 100% identity values and 0.0 E values (Table 5.1). Four different species were identified: *Metarhizium anisopliae* (MF, MR, CR, S4ST7 and ICIPE 30), *Beauveria bassiana* (BF and ICIPE 279), *Phanerochaete chrysosporium* (ZF) and *Epacris microphylla* (ZRA) (Table 5.1). This confirmed the morphological identifications.

Table 5.1: Identified fungal endophytes species using ITS 5 and 4 and AB28 and TW81

Sample codes	Length (base pair)	Accession number	Gene identified from GeneBank at the NCBI database	E value	% Identities
ITS 5 and 4					
ICIPE30	541	FJ545302.1	***Metarhizium anisopliae*** isolate CNXJ2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
S4ST7	541	FJ545302.1	***Metarhizium anisopliae*** (same isolate CNXJ2 18S ribosomal RNA gene as described above)	0	99
MF	541	FJ545302.1	***Metarhizium anisopliae*** (same isolate CNXJ2 18S ribosomal RNA gene as described above)	0	99
MR	541	FJ545302.1	***Metarhizium anisopliae*** (same isolate CNXJ2 18S ribosomal RNA gene as described above)	0	99
CR	541	FJ545302.1	***Metarhizium anisopliae*** (same isolate CNXJ2 18S ribosomal RNA gene as described above)	0	99
ICIPE 279	551	JQ266208.1	***Beauveria bassiana*** strain MTCC_6286 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
BF	551	AJ560668.1	***Beauveria bassiana*** ITS1, 5.8S rRNA gene and ITS2, isolate IMI 386701	0	100
ZF	621	GU966518.1	***Phanerochaete chrysosporium*** strain TS03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
ZRA	538	AY268204.1	***Epacris microphylla*** root associated fungus 20 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	0	99

AB28 and TW81					
ICIPE 30	507	FJ545302.1	***Metarhizium anisopliae*** isolate CNXJ2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
S4ST7	507	FJ609312.1	***Metarhizium anisopliae*** strain M1311 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, partial sequence	0	100
MF	507	FJ609312.1	***Metarhizium anisopliae*** strain M1311 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	100
MR	507	FJ609312.1	***Metarhizium anisopliae*** strain M1311 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	100
CR	507	FJ609312.1	***Metarhizium anisopliae*** strain M1311 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	100
ICIPE 279	553	JQ999974.1	***Beauveria bassiana*** strain YNSK1106 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
BF	517	AJ560668.1	***Beauveria bassiana*** ITS1, 5.8S rRNA gene and ITS2, isolate IMI 386701	0	100
ZF	587	GU966518.1	***Phanerochaete chrysosporium*** strain TS03 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	0	99
ZRA	504	AY268204.1	***Epacris microphylla*** root associated fungus 20 18S ribosomal RNA gene, partial sequence; and internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	0	99

5.2.3 Phylogenetic trees

Phylogenetic tree using ITS 5 and 4 regions

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 486.25000000 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figure 5.6). The evolutionary distances were computed using the number of differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 494 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

This analysis clustered all the isolates included in the study into four groups as expected (Figure 5.6). The first group had the clustering of the *Metarhizium anisopliae* isolates complex where the isolates MR, MF and CR branched separately from the standards included in the study i.e. ICIPE30 and S4ST7. The second group consisted of *Beauveria bassiana* isolates BF and ICIPE 279 branching from the same node but occupying different branches, a clear indication that BF which was isolated from *V. faba* seeds is different from the standard ICIPE 279 isolate that was isolated from Coleopteran larvae. With ITS 5 & 4 region, all the *Metarhizium* isolates analysed the identity linked to a *Metarhizium* spp. of accession number FJ545302.1 (Table 5.1). The last two clusters consisted of *Epacris microphylla* isolate ZRA and *Phanerochaete chrysosporium* isolate ZF, respectively (Figure 5.6).

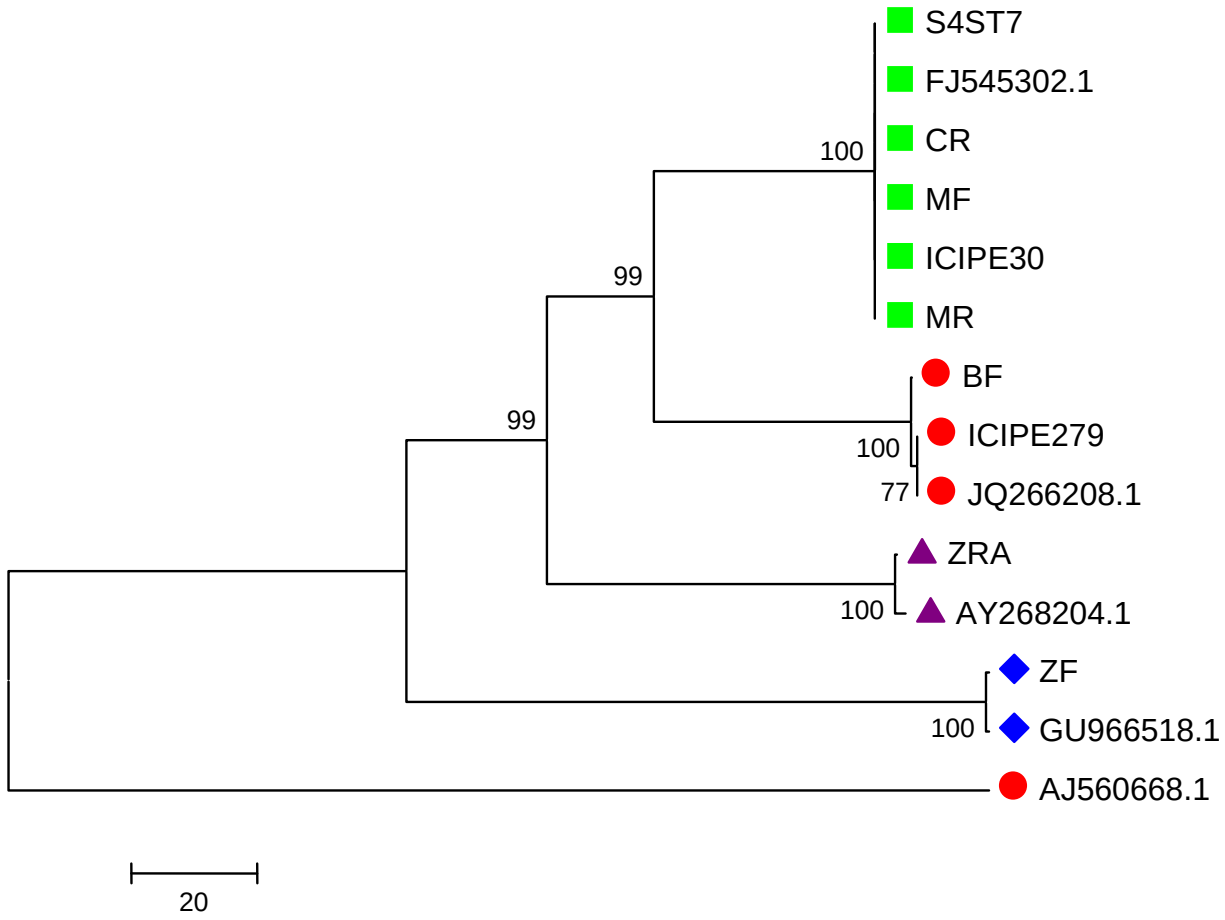

Figure 5.6: Phylogenetic tree using ITS 5 and 4 regions showing the evolutionary relationships of fungal endophyte isolates

The table of genetic distances (Table 5.2) constructed by Mega 5 (Tamura *et al*., 2011) using the Kimura 2-parameter model, was used to generate principal component plots, using the GenAlEx 6.41 program (Peakall and Smouse, 2006). In this analysis of the fungal endophytes species using principal coordinate analysis (PCA), the first two axes explained 62.84% of the variation (the first axis 36.67%, and the second axis 26.17%) (Figure 5.7). The PCA separated the nine isolates into four distinct clusters. Each cluster was occupied by the isolates belonging to the different genera i.e. *Metarhizium* sp., *Beauveria* sp., *Epacris microphylla* and *Phanerochaete chrysosporium* respectively (Figure 5.7).

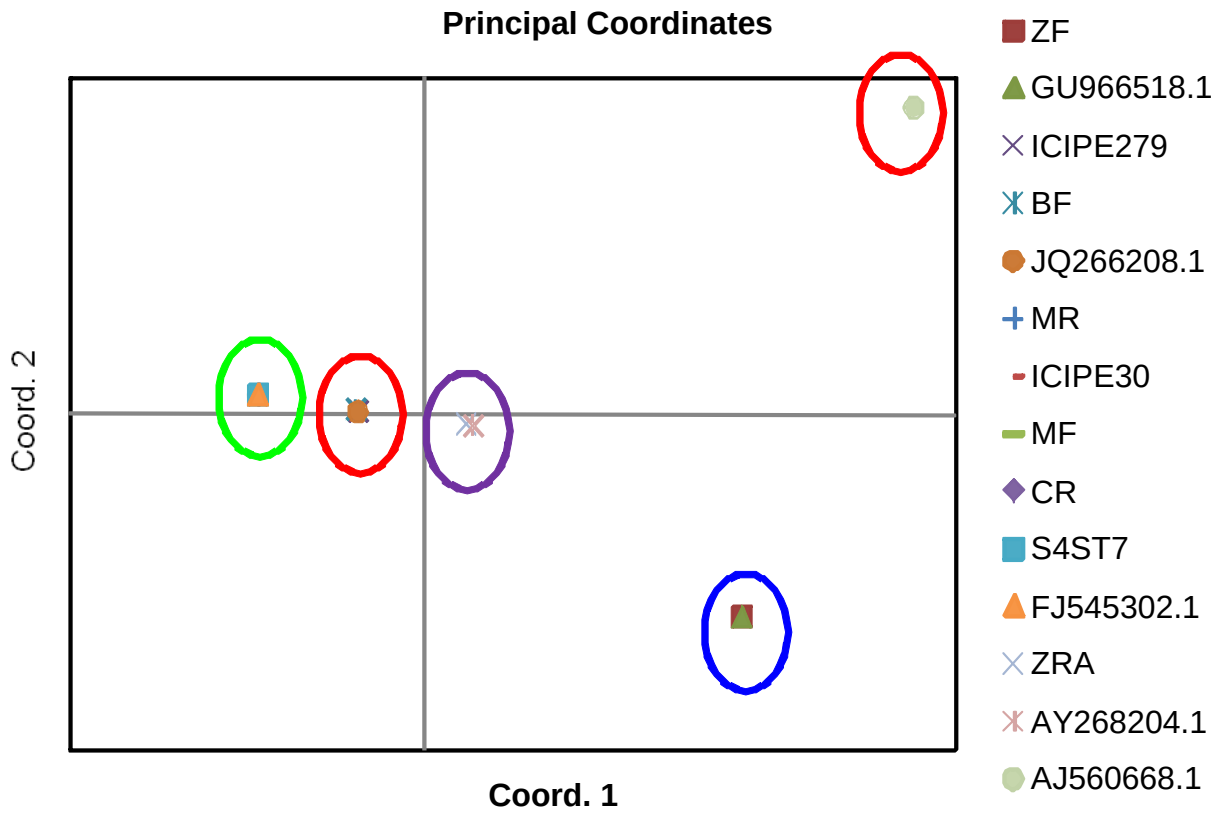

Figure 5.7: Plot of principal components analysis (PCA) via the covariance matrix with data standardization calculated using GenAIEx for the various endophytes species when using ITS 5 & 4 regions.

The estimates of evolutionary divergence between sequences of the various endophyte isolates when using ITS 5 & 4 ranged between 0.0 and 312.0 (Table 5.2). Comparison of *Metarhizium anisopliae* isolates (MF and MR) isolated from *V. faba* and *P. vulgaris* with the standards ICIPE 30 and S4ST7, gave a square distance of 0.0. Same trend was observed with CR. However, comparison of *Beauveria bassiana* isolate BF isolated from *V. faba* with the standards ICIPE 279, gave a square distance of 1.0 (Table 5.2).

Table 5.2: Estimates of evolutionary divergence between sequences using ITS 5 & 4

Fungal Isolates	ZF	GU966518.1	ICIPE279	BF	JQ266208.1	MR	ICIPE30	MF	CR	S4ST7	FJ545302.1	ZRA	AY268204.1	AJ560668.1
ZF	-													
GU966518.1	1.0	-												
ICIPE279	171.0	171.0	-											
BF	172.0	172.0	1.0	-										
JQ266208.1	171.0	171.0	0.0	1.0	-									
MR	167.0	167.0	77.0	76.0	77.0	-								
ICIPE30	167.0	167.0	77.0	76.0	77.0	0.0	-							
MF	167.0	167.0	77.0	76.0	77.0	0.0	0.0	-						
CR	167.0	167.0	77.0	76.0	77.0	0.0	0.0	0.0	-					
S4ST7	167.0	167.0	77.0	76.0	77.0	0.0	0.0	0.0	0.0	-				
FJ545302.1	167.0	167.0	77.0	76.0	77.0	0.0	0.0	0.0	0.0	0.0	-			
ZRA	175.0	175.0	117.0	116.0	117.0	107.0	107.0	107.0	107.0	107.0	107.0	-		
AY268204.1	173.0	173.0	119.0	118.0	119.0	109.0	109.0	109.0	109.0	109.0	109.0	2.0	-	
AJ560668.1	312.0	312.0	297.0	296.0	297.0	295.0	295.0	295.0	295.0	295.0	295.0	296.0	295.0	-

Phylogenetic tree using AB28 and TW81 regions

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 465.81250000 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, (1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figure 5.8). The evolutionary distances were computed using the number of differences method (Nei and Kumar 2000) and are in the units of the number of base differences per sequence. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 444 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011).

This analysis clustered also the *Metarhizium* sp.*, Phanerochaete chrysosporium*, *Epacris microphylla* and *Beauveria* sp. isolates into four different groups (Figure 5.8). The first cluster consisted of the *Metarhizium anisopliae* isolates analysed. The MR, MF and CR isolates branched separately from the standards (ICIPE30 and S4ST7) included in the study. The second group consisted of *Beauveria bassiana* isolates BF though it branched separately from the standard ICIPE 279 in spite belonging to the same genus and species. This clearly shows that the BF isolated from *V. faba* seeds is different from the standard isolate ICIPE 279. The last two clusters consisted of the *Epacris microphylla* ZRA and *Phanerochaete chrysosporium* ZF, respectively (Figure 5.8).

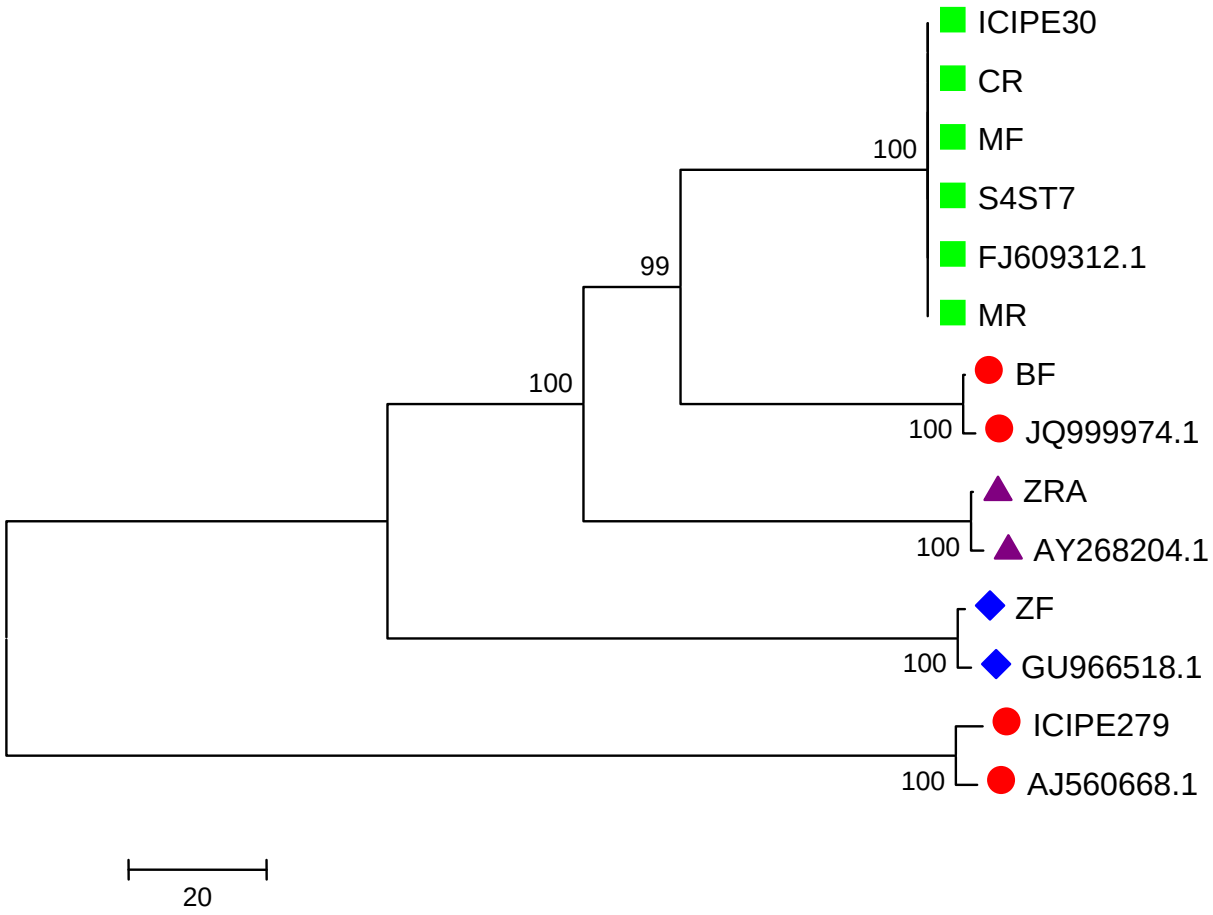

Figure 5.8: Phylogenetic tree using AB28 and TW81 regions showing the evolutionary relationships of fungal endophyte isolates.

The table of genetic distances (Table 5.3) constructed by Mega 5 (Tamura *et al*., 2011) using the Kimura 2-parameter model, was used to generate principal component plots, using the GenAlEx 6.41 program (Peakall and Smouse, 2006). In this analysis of the fungal endophytes species using principal coordinate analysis (PCA), the first two axes explained 71.78% of the variation (the first axis 46.87%, and the second axis 24.90%) (Figure 5.9). The PCA separated the nine isolates into four distinct clusters as observed in ITS 5 & 4 case. A cluster was occupied by the genera belonging to *Metarhizium*, a cluster consisting of *Beauveria*, and *Epacris* and *Phanerochaete* on their own clusters (Figure 5.9).

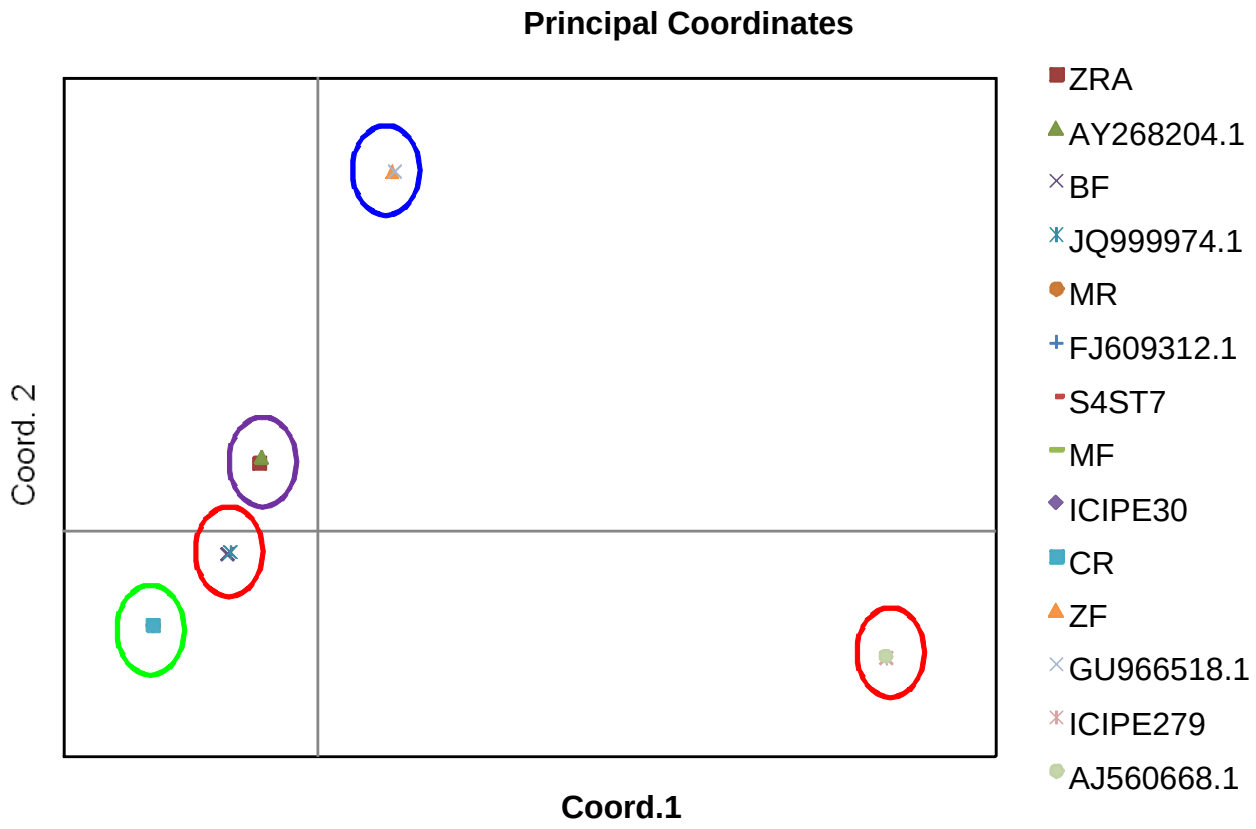

Figure 5.9: Plot of principal components analysis (PCA) via the covariance matrix with data standardization calculated using GenAIEx for the various endophytes species when using AB28 & TW81 regions.

The estimates evolutionary divergence between sequences of the various endophyte isolates when using AB28 & TW81 primer region ranged between 0.0 and 285.0 (Table 5.3). Comparison of *Metarhizium anisopliae* isolates (MF and MR) isolated from *V. faba* and *P. vulgaris* with the standards ICIPE 30 and S4ST7, gave a square distance of 0.0. Same trend was observed with CR. However, comparison of *Beauveria bassiana* isolate BF isolated from *V. faba* with the standard ICIPE 279, gave a square distance of 276.0 (Table 5.3).

Table 5.3: Estimates of evolutionary divergence between sequences using AB28 & TW81

Fungal Isolates	ZRA	AY268204.1	BF	JQ999974.1	MR	FJ609312.1	S4ST7	MF	ICIPE30	CR	ZF	GU966518.1	ICIPE279	AJ560668.1
ZRA	-													
AY268204.1	2.0	-												
BF	113.0	115.0	-											
JQ999974.1	115.0	117.0	2.0	-										
MR	106.0	108.0	77.0	79.0	-									
FJ609312.1	106.0	108.0	77.0	79.0	0.0	-								
S4ST7	106.0	108.0	77.0	79.0	0.0	0.0	-							
MF	106.0	108.0	77.0	79.0	0.0	0.0	0.0	-						
ICIPE30	106.0	108.0	77.0	79.0	0.0	0.0	0.0	0.0	-					
CR	106.0	108.0	77.0	79.0	0.0	0.0	0.0	0.0	0.0	-				
ZF	170.0	168.0	171.0	171.0	162.0	162.0	162.0	162.0	162.0	162.0	-			
GU966518.1	170.0	168.0	173.0	173.0	163.0	163.0	163.0	163.0	163.0	163.0	3.0	-		
ICIPE279	284.0	285.0	276.0	276.0	276.0	276.0	276.0	276.0	276.0	276.0	282.0	281.0	-	
AJ560668.1	282.0	283.0	274.0	274.0	276.0	276.0	276.0	276.0	276.0	276.0	281.0	280.0	7.0	-

5.3 Discussion

All the fungal species identified using morphological tools, were confirmed with molecular identification. Results from the two gene regions utilized yielded similar results, confirming that the morphological identification of the isolates are in line with the molecular identification and indeed the endophytes belong to the four fungal genera of *Metarhizium*, *Beauveria*, *Epacris* and *Phanerochaete.* Both the two gene regions linked the CR, MR and MF and the *icipe* standards to the *Metarhizium anisopliae* spp. though they occupied different branches from the standards. While the BF linked to the *Beauveria* spp. as expected and also branched differently from the *icipe Beauveria* standard. In a study, Akello (2012) reported isolate S4ST7 as *M. anisopliae* species using molecular characterization. At the difference of ITS 4 & 5 and AB28 & TW81 used in this study, the author used IGS [PNFo (CCCGCCTGGCTGCGTCCGACTC) and PN22 (CAAGCATATGACTACTGGC)] and ITS [ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC)] primers. This confirms our result of S4ST7 using ITS 5 & 4 and AB28 & TW81 regions.

In addition, *Beauveria bassiana* ICIPE 279 was reported endophytic in *V. faba* and *P. vulgaris* by Akutse *et al*. (2013) and since BF belongs to the same cluster as ICIPE 279 and was isolated from *V. faba* seeds, it may also be endophytic not just for the seeds of *V. faba* but also in the plants when inoculated. The presence of these endophytes in *V. faba* and *P. vulgaris* seeds shows a high chance that the seedlings of these seeds may be colonized by the same fungal endophytes either naturally or artificially through inoculation. The results excluded possible effects of competition / interference since other microorganisms were not present in the sterilized soil as compared to in the natural field conditions where the soil will not be sterilized (Akutse *et al*., 2013). Similarly, Aldrich-Markham *et al*. (2007) reported that seeds contained endophytes and used percentage of seeds infected to test the possible transmission of these endophytes from seeds to seedlings/plants. For example, a 25 percent level means that 25 out of 100 seeds contain endophytes and will produce colonized plants. The *Epacris microphylla* which was identified as an endophyte in this study was also identified during a survey of the endophytic fungi associated with the Chinese traditional medicinal plant

Achyranthes bidentata Blume (Amaranthaceae) by Bing-Da *et al*. (2013) as a new Ascomycete species of endophytic fungal from the stem of this plant (*E. achyranthi*). In addition, we can say that the endophytic fungi isolated from *V. faba* and *P. vulgaris* seeds, may accumulate naturally inside the seeds in the farm or during their processing.

5.4 Conclusion

Beauveria bassiana isolate BF, *M. anisopliae* isolates MF, MR, *Phanerochaete chrysosporium* ZF and *Epacris microphylla* ZRA were found as seed fungal endophytes while *M. anisopliae* CR was identified as a *P. vulgaris* root endophyte. They were clearly different from the standards *B. bassiana* ICIPE279 and *M. anisopliae* ICIPE279 and S4ST7 thus clustering into the groups, respectively. The advantages of this characterization is that it will help the scientific world not only in awareness about seed endophytes but also to see if the presence of these fungi will (i) inhibit/suppress the endophytes growth/colonization which will be artificially inoculated (specially through seed inoculation) inside the host plants for pests control in general and *Liriomyza* leafminer species particularly, (ii) have any additive, symbiotic or synergic effects in the management of *Liriomyza* leafminers and other pests, (iii) test also pathogenicity and virulence of these seed endophytes on different sucking arthropods *in vitro* and *in vivo*, (iv) assess the quality of the seeds in the presence of these endophytes, (v) assess the nutritional value of the seeds containing the endophytes and finally (vi) assess the effects of the endophytes on stored products insects when storing seeds derived from endophytically inoculated plants.

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CHAPTER 6: EFFECTS OF ENDOPHYTICALLY-COLONIZED *VICIA FABA* (FABACEAE) ON THE LIFE-HISTORY PARAMETERS OF PARASITOIDS *PHAEDROTOMA SCABRIVENTRIS* NIXON (HYMENOPTERA: BRACONIDAE) AND *DIGLYPHUS ISAEA* WALKER (HYMENOPTERA: EULOPHIDAE)

Abstract

The tritrophic interaction between fungal endophytes *Beauveria bassiana* isolates ICIPE 279, G1LU3, S4SU1 and *Hypocrea lixii* isolate F3ST1, which were found pathogenic to *Liriomyza huidobrensis*, *Vicia faba* and parasitoids was studied. The effects of endophytically-colonized fungal pathogens on mortality, adult emergence and survival of *Phaedrotoma scabriventris* and *Diglyphus isaea* were investigated after endophytic colonization of *V. faba* plants and infested with *Liriomyza* larvae. All the fungal isolates were pathogenic to *L. huidobrensis,* by reducing their oviposition ability, and causing larval and pupal mortality. However, no significant difference was observed between the control and the endophytically-treated plants in terms of parasitism rates of *P. scabriventris* ($p = 0.68$) and *D. isaea* ($p = 0.45$) and the exposed adult parents' survival times ($p = 0.06$). In addition, the survival period of the progeny F1 of *P. scabriventris* was slightly reduced by *B. bassiana* S4SU1 to 28 days as compared to the rest of the endophyte isolates, but no significance difference ($p = 0.54$) was observed on the survival times of the progeny F1 of *D. isaea*. There was a complementarity effects observed between the endophytes and the parasitoids performance, since the studied endophyte isolates of *B. bassiana* and *H. lixii* were able to negatively affect the host *L. huidobrensis* without been harmful to the parasitoids.

6.0 Introduction

Horticultural crops represent the most important economical sector of agriculture in Kenya. However, it is limited by a number of pests notably arthropods. Among these pests, are the invasive leaf miner species: *Liriomyza huidobrensis* (Blanchard), *L. sativae* Blanchard and *L. trifolii* (Burgess) (Diptera: Agromyzidae) that pose the greatest

challenge as they damage major vegetable and ornamental crops, serve as vector for diseases and constitute quarantine pests in European markets (Johnson *et al*., 1983; Parrella *et al*., 1984; Deadman *et al.,* 2002; Chabi-Olaye *et al*., 2008; EPPO, 2009). *Liriomyza* leafminers are therefore economically important pests of horticultural crops (Spencer, 1973; Murphy and LaSalle, 1999).

The management of leafminers worldwide, and particularly in East Africa, has commonly relied on the frequent use of synthetic chemical insecticides (Chandler, 1981, 1984; MacDonald, 1991; Gitonga *et al*., 2010). However, the indiscriminate and frequent use of these chemicals resulted in insecticide resistance of flies (Parella *et al*., 1984; Murphy and LaSalle, 1999), pollution of the environment as well as elimination of their natural enemies (Johnson *et al*., 1980; Murphy and LaSalle, 1999). Chemical control is also not very effective since flies usually escape insecticide applications due to their high mobility. Furthermore, *Liriomyza* larvae are inaccessible to many pesticides because they develop inside leaves and pupate in soil (Mujica and Kroschel, 2011). Horticultural producers are also under pressure since the introduction of maximum residue level (MRL) set up by the European Union on export produce. This has led to an upsurge in research aimed at more biorational management alternatives. Biological control using parasitoids, entomopathogenic fungi and endophytes is being considered as alternative to leafminer management in East Africa (Migiro *et al*., 2010, Akutse *et al*., 2013). *Hypocrea lixii* isolate F3ST1 and *Beauveria bassiana* isolates G1LU3, S4SU1 and ICIPE 279 were found endophytic in *Vicia faba* and *Phaseolus vulgaris* and were the most pathogenic and virulent isolates against *L. huidobrensis* in causing mortality, reducing adult survival, number of pupae, progeny survival and adult emergence (Akutse *et al*., 2013).

The use of parasitoids and endophytic fungi are of paramount importance in a control strategy of the pests and their interactions become a key interest of understanding. Tritrophic interactions between plant secondary (or fungal endophyte induced) chemicals, insect herbivores, and parasitoids are common in natural and managed systems (Price *et al*., 1980; Barbosa and Saunders, 1985; Turlings and Benrey, 1998; Heil, 2008). Parasitoids may benefit by exploiting constitutive or induced phytochemicals as kairomones in host finding (Nordlund *et al*., 1988; Turlings *et al*.,

1992; De Moraes *et al*., 1998; Turlings and Benrey, 1998; Fukushima *et al*., 2002; Heil 2008) or their fitness may be reduced when hosts are stunted from the metabolic stress of coping with ingested phytochemicals (Slansky, 1986), or when larval parasitoids encounter such toxins in the bodies of their hosts (Campbell and Duffey 1979; Duffey *et al*., 1986; Price 1986; Thorpe and Barbosa 1986; Barbosa *et al*., 1991; Gauld and Gaston, 1994; Lampert and Bowers, 2010). Understanding how different parasitoids are affected by such interactions is important for integrating plant resistance and biological control in pest management (Campbell and Duffy, 1979, Price *et al*., 1980).

The objective of this study was therefore to investigate the tritrophic interactions between the main biocontrol agents, the above mentioned fungal endophytes, the ectoparasitoid *Diglyphus isaea* and the endoparasitoid *Phaedrotoma scabriventris.*

6.1 Materials and methods

6.1.1 Fungal cultures

Beauveria bassiana isolates G1LU3, S4SU1 and ICIPE 279, and *Hypocrea lixii* isolate F3ST1, previously reported pathogenic to *L. huidobrensis* (Chapter 3), were used in this study. *Beauveria bassiana* isolates G1LU3, S4SU1 and *H. lixii* isolate F3ST1 were isolated from the aboveground parts of maize and maize and sorghum e respectively (Akello, 2012) while *B. bassiana* isolate ICIPE279 was isolated from a Coleopteran larva. The isolates were cultured on potato dextrose agar (PDA), and were maintained at 25 ± 2 ^{o}C in complete darkness. Conidia were harvested by scraping the surface of 2-3-week old sporulating cultures with a sterile spatula. 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. Conidial counts were done using a Neubauer Hemacytometer (Goettel and Inglis, 1997). The conidial suspension was adjusted to 1 x 10^8 conidia ml^{-1} through dilution prior to inoculation of seeds.

Spore viability was determined before any bioassay by plating 0.1 ml of 3 x10^6 conidia ml^{-1} onto 9-cm Petri dishes containing 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 ^{o}C and were 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) using the method described by Goettel and Inglis, (1997). Conidia were deemed to have germinated when the length of the germ tube was approximately two times the diameter of the propagule / conidium. Four replicates were used for each isolate.

6.1.2 Plant inoculation and colonization of endophyte isolates

Inoculation was done by soaking seeds of *V. faba* (a local Kenyan Open Pollinated variety) in conidial suspensions titrated at 10^8 ml^{-1} 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 (Schultz *et al.,* 1998). Seeds were transferred into plastic pots (8 cm diameter x 7.5 cm high) containing the planting substrate (mixture of manure and soil 1:5). The substrate was sterilized in an autoclave for 2 hours at 121 ^oC and allowed to cool for 72 hours prior to planting. Five seeds were sowed per pot and maintained at room temperature (25 ± 3 ^oC and 60% RH). Pots were transferred immediately after germination to a screen house (2.8 m length x 1.8 m width x 2.2 m height) at 25 ± 3 ^oC, for two weeks. Seedlings were thinned to three per pot after germination and were watered twice per day (morning and afternoon). No additional fertilizer was added to the substrate.

6.1.3 Insects

Liriomyza huidobrensis

Liriomyza huidobrensis was obtained from the Animal Rearing and Quarantine Unit (ARQU), of International Centre of Insect Physiology and Ecology (*icipe*). The initial colony originated from adult leafminers collected from wild crucifers on the *icipe* campus (01º13.3′S 36º53.8′E, 1600 m a.s.l.) and was reared on *V. faba* for 8 - 10 generations prior to experiments. Rearing colonies were maintained at 27 ± 2 ºC with a photoperiod of 12L: 12D and relative humidity of approximately 40%. *L. huidobrensis* adults were fed on a 10 % sucrose solution.

Diglyphus isaea

The ectoparasitoid *D. isaea* used in the experiments was also obtained from the ARQU, *icipe*. The colony originated from adult *D. isaea* collected from leafminer-infested French bean, tomatoes and crucifers at Naivasha (S: 00.66731^{o}; E: 036.38603^{o}; Elevation: 1906 m), Kenya. *Diglyphus isaea* was reared on *L. huidobrensis*-infested *V. faba* in Plexiglas cages (50 cm x 50 cm x 45 cm) for 5 - 10 generations prior to experiments. For the rearing of parasitoids, *D. isaea* adults were exposed to 2nd and 3rd-instar larvae of *L. huidobrensis* and the colony was maintained at 27 ± 2 ºC with a photoperiod of 12L: 12D and 40-50% rh.

Phaedrotoma scabriventris

The initial colony of *P. scabriventris* originated from Peru and was maintained in the quarantine facilities of *icipe* on 2nd and 3rd instar *L. huidobrensis* larvae-infested *V. faba* host plants for 5 - 10 generations prior to experiments

Phaedrotoma scabriventris and *D. isaea* adults were fed on a 10 % honey solution prior to exposure during their given mating period, 24 hours post-emergence.

6.1.4 Effects of endophytically-colonized *Vicia faba* host plants on "life history" of *Phaedrotoma scabriventris* and *Diglyphus isaea*

The effect of *V. faba* endophytically-colonized by fungal endophytes on larval and pupal mortality, fly emergence and survival of *P. scabriventris and D. isaea* adults was evaluated in inoculated plants. In order to obtain leafminer-infested plants with larvae of the appropriate size (2nd and 3rd instars), two-day old mated adult flies (150 individuals at sex ratio of 1: 2 male: female) were exposed for 48 hours to two week-old endophyte-inoculated host plant seedlings in Plexiglas cages (50 cm x 50 cm x 45 cm). Each cage contained five potted plants and represented a treatment and was maintained at 25–27 ^{o}C, at 50–70% RH and 12L: 12D photoperiod. All the treatments were arranged in a randomized complete block design and the experiment replicated three times over time. After 48 hours post-exposure, flies were removed from the cages using mouth

aspirators to prevent excessive oviposition and feeding damage by adult flies. The inoculated-exposed plants were maintained until larvae reached the 2nd and 3rd instars (approximately 5–8 days post-exposure). In the control, plants were not inoculated with fungal pathogens.

The endophytically-inoculated *V. faba* plants infested with 2nd and 3rd instar larvae were used for parasitoids exposure. Fifty adult *P. scabriventris* (in the sex ratio of 1: 2, males: females) and 50 adult *D. isaea* (in the sex ratio of 1: 2, males: females) were exposed separately to endophytically-inoculated infested plants with 2nd and 3rd instar larvae for 48 hours after which the exposed plants were removed and held for parasitoids pupal development.

Survival of exposed adult parasitoids was recorded by counting daily the number of live parasitoids inside the cages until all parasitoids had died. Dead parasitoids were placed on Petri dishes lined with damp sterilized filter paper to allow fungal growth on the surface of the cadaver (mycosis test). Pupae were harvested from leaves 3 -5 days post-exposure to parasitoids, counted and then incubated at 25 ± 1 ^{o}C. Adult emergence of both parasitoids and flies and sex ratio were determined and parasitism rates were calculated. In order to determine adult survival, 20 adult parasitoids were selected from the above experiment (progenies) and their mortality/survival recorded daily until all parasitoids died. The parasitoids were maintained in a cage as described above. A 10% honey solution was provided as food and cages maintained at 25 ±1 ^{o}C.

6.1.5 Statistical analyses

Mortality, number of pupae, emergence and survival (for parent parasitoids and F1 progeny) data were analyzed using both analysis of variance (ANOVA) and survival analysis techniques. The survival curves were generated using the Kaplan-Meir (K-M) method. The log-rank test was used to compare the effect of various isolates on survival of *P. scabriventris and D. isaea*.

The K-M estimator of the survivor function was:

$$\widehat{S}(t) = \prod_{y_{(i)} \leq t} \widehat{p_i} = \prod_{y_{(i)} \leq t} \left(\frac{n_i - d_i}{n_i} \right) = \prod_{i=1}^{k} \left(\frac{n_i - d_i}{n_i} \right),$$

where $y_{(k)} \leq t < y_{(k+1)}$, n_i = the number in the risk set just before time *t*, d_i = number died at time $y_{(i)}$, p_i = probability of surviving through the interval given being alive at the beginning of the interval, and *y*(*i*) denotes the *i*th distinct ordered censored or uncensored observation.

The number of pupae was log-transformed [Log_{10} (x + 1)] before ANOVA analysis while the emergence and parasitism rates were square root-transformed [√(x + 1)] before applying ANOVA analysis. Tukey HSD multiple comparisons of means was used to separate the means. The success rate (%) of parasitism was calculated as follows:

$$\% \text{ parasitism} = \frac{\text{Number of emerged parasitoid species}}{\text{Total number of emerged parasitoids and flies}} \times 100$$

All the analyses were performed using R (2.13.1) statistical software (R Development Core Team, 2011) while relying heavily on the epicalc package (Chongsuvivatwong, 2012).

6.2 Results

6.2.1 Effects of endophytically-colonized *Vicia faba* host plant on parasitism rates of *Diglyphus isaea* and *Phaedrotoma scabriventris*

The parasitism rates of *D. isaea* and *P. scabriventris* ranged between 15 – 35 % and 56 – 64% respectively (Table 6.1). No significance differences were observed between the control and the endophytically-treated plants in terms of parasitism rates of *P. scabriventris* (*F = 0.59, df = 4, 9, p = 0.68*) and *D. isaea* (*F = 1.02, df = 4, 9, p = 0.45*) (Table 6.1). For example, the parasitism rate observed in *H. lixii* F3ST1 (63.6 ± 5.6) was similar to the one observed in the control (62.4 ± 1.3) for *P. scabriventris*. The same

trend was observed for *D. isaea* in *H. lixii* F3ST1 (33.4 ± 12.8) and in the control (34.6 ± 0.2) respectively (Table 6.1).

Table 6.1: Effects of endophytically-treated hosts on the parasitism of *Diglyphus isaea* and *Phaedrotoma scabriventris*

	% Parasitism	
Fungal endophyte isolates	***Diglyphus isaea***	***Phaedrotoma scabriventris***
Beauveria bassiana S4SU1	16.3 ± 9.3 [a]	55.7 ± 1.7 [a]
Beauveria bassiana ICIPE279	15.5 ± 8.9 [a]	62.2 ± 1.9 [a]
Beauveria bassiana G1LU3	14.8 ± 8.3 [a]	57.1 ± 7.4 [a]
Hypocrea lixii F3ST1	33.4 ± 12.8 [a]	63.6 ± 5.6 [a]
Control	34.6 ± 0.2 [a]	62.4 ± 1.3 [a]

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

6.2.2 Effects of endophytically-colonized *Vicia faba* host plant on life- parameters of *Diglyphus isaea* and *Phaedrotoma scabriventris*

6.2.2.1 *Diglyphus isaea* adult survival

The survival time of the exposed adults of *D. isaea* to the different endophytically-inoculated and infested *V. faba* was not significantly reduced (*F = 2.3, df = 4, 555, p = 0.056*) for *D. isaea* in the treatments as compared to the control (Figure 6.1).

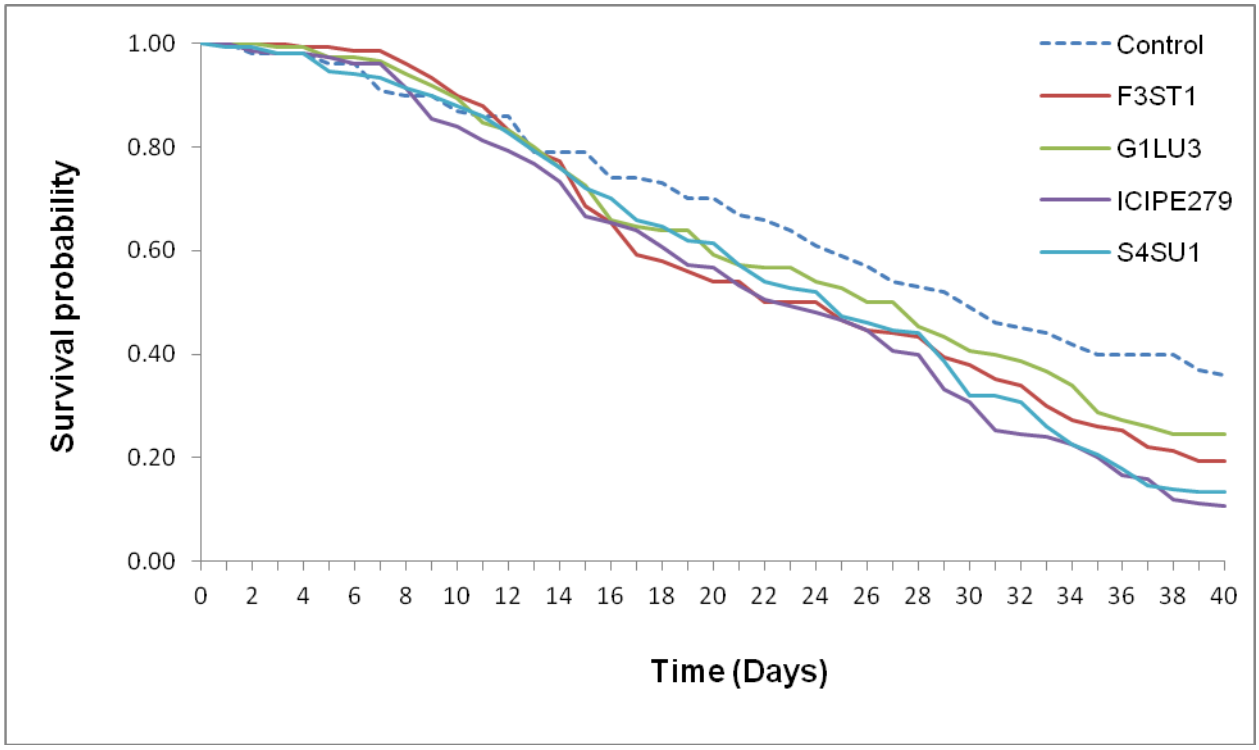

Figure 6.1: Survival curves for *Diglyphus isaea* adults following exposure to *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* F3ST1 and infested with 2nd and 3rd instar larvae of *Liriomyza huidobrensis* 40 days after exposure.

The survival of adult *D. isaea* exposed to endophytically-inoculated and infested hosts varied among the treatments (*proximate log rank test = 19.48, df = 4, p < 0.0001*). This difference was mainly due to the control which had slightly higher survival as compared to other treatments which followed uniformly the same trends (Figure 6.1). For example, at 7 days post-exposure, survival was above 90% for *D. isaea* in the control as well as in the treatments. After 14 days, the survival was above 75% for all the treatments and in the control and remained above 50% at 21 days for all the treatments and the control (Figure 6.1). However, at 29 days, the survival was 52.0% in the control and dropped to 39.3%, 43.3%, 33.3%, 38.7% and 39.3% in *B. bassiana* G1LU3, ICIPE279, S4SU1 and in *H. lixii* respectively (Figure 6.1). At 40 days, where the survival was 36.0% in the control, it was reduced to 10.7%, 13.3%, 24.7% and 19.3% in *B. bassiana* ICIPE279, S4SU1, G1LU3 and in *H. lixii* F3ST1 respectively (Figure 6.1).

6.2.2.2 *Phaedrotoma scabriventris* adult survival

The survival of adult *P. scabriventris* exposed to endophytically-inoculated and infested plants varied among the treatments (*proximate log rank test = 26.32, df = 4, p < 0.0001*). This difference was mainly due to the control which always has slightly high survival rates within times as compared to the treatments (Figure 6.2).

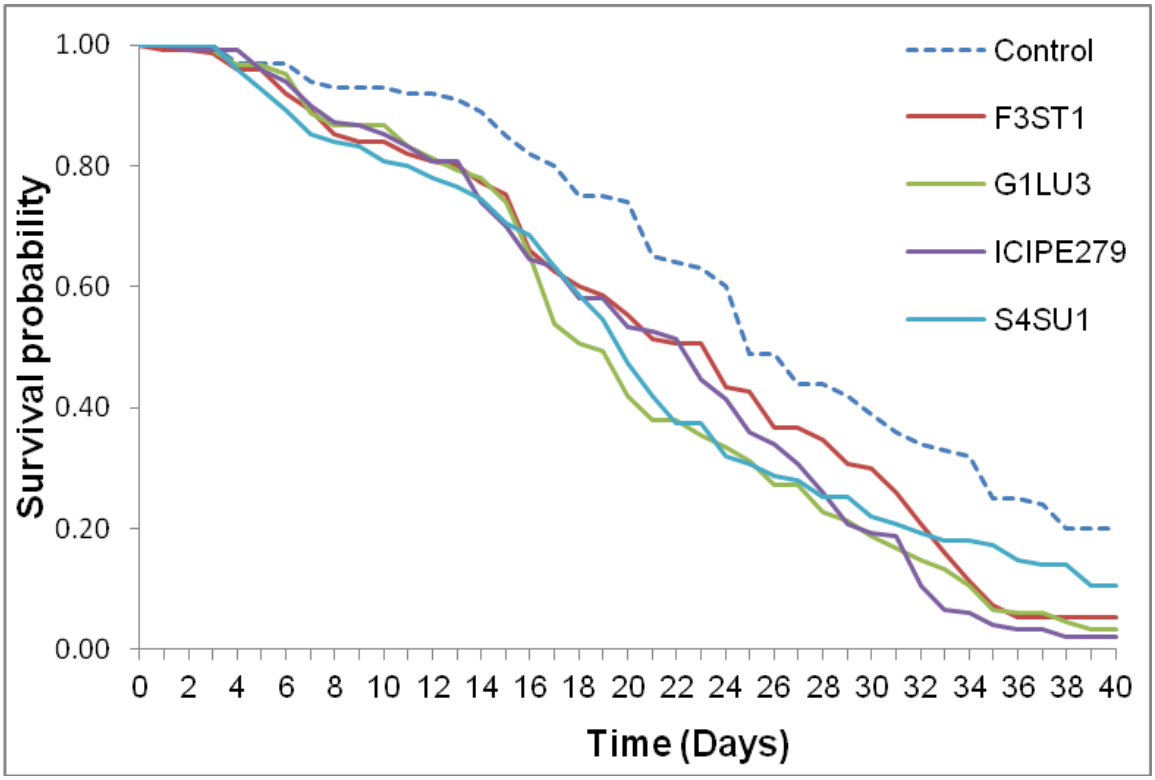

Figure 6.2: Survival curves for *Phaedrotoma scabriventris* adults following exposure to *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* F3ST1 and infested with 2nd and 3rd instar larvae of *Liriomyza huidobrensis* 40 days after exposure.

For example, at 7 days post-exposure, survival was above 90% for *P. scabriventris* in the control as well as in the treatments. At 14 days, the survival was above 75% for all the treatments and in the control. At 21 days when the survival was at 65.0% in the control, it was reduced to 38.0% and 42.0% in *B. bassiana* G1LU3 and S4SU1 respectively. However, at 24 days, the survival was at 60.0% and 32.0% in the control and *B. bassiana* S4SU1 respectively. At 40 days, where the survival was 20.0% in the

control, it was significantly reduced to 10.7%, 3.3%, 2.0% and 5.3% in *B. bassiana* S4SU1, G1LU3, ICIPE 279 and in *H. lixii* F3ST1 respectively (Figure 6.2).

When considering the survival time at 40 days, the exposed population of *P. scabriventris* was more reduced as compared to *D. isaea* in all the treatments within times. There was no significant difference ($F = 0.59, df = 4, 9, p = 0.675$) between the control and the treatments for *D. isaea* (Table 6.2). However, there was significant difference ($F = 23.64, df = 4, 9, p < 0.0001$) in the mean survival times (median times) of *P. scabriventris* among the treatments and the control (Table 6.2). For example, when the mean survival time was 26.0 ± 1.0 days in the control, it was at 12.7 ± 0.9 days in *B. bassiana* ICIPE 279 for *P. scabriventris*. *Beauveria bassiana* ICIPE 279 reduced *P. scabriventris* exposed adults' population levels as compared to the control and other endophyte isolates (Table 6.2).

Table 6.2: Mean survival period of *Diglyphus isaea* and *Phaedrotoma scabriventris* parent adults exposed to infested-*Vicia faba* plants endophytically-colonized by the different endophytic fungal isolates

Fungal isolates species	**Mean survival time (Days) ± SE**	
	Diglyphus isaea	***Phaedrotoma scabriventris***
Beauveria bassiana ICIPE 279	21.2 ± 3.2 [a]	12.7 ± 0.9 [c]
Beauveria bassiana G1LU3	24.3 ± 3.1 [a]	18.5 ± 1.0 [b]
Beauveria bassiana S4SU1	22.3 ± 3.2 [a]	19.5 ± 0.3 [b]
Hypocrea lixii F3ST1	24.5 ± 4.8 [a]	21.5 ± 1.2 [ab]
Control	29.3 ± 2.7 [a]	26.0 ± 1.0 [a]

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

6.2.2.3 Pupation after exposure of *Diglyphus isaea*

Fewer pupae were produced in endophytically-colonized plants than in the control (423.5 ± 3.5 pupae) (Figure 6.3). There were, however, significant differences ($F = 30.40, df = 4, 9, p < 0.0001$) among the fungal isolates and the control. For example, the number of pupae produced in *B. bassiana* G1LU3 (307.0 ± 10.3) was significantly different from the control and *H. lixii* F3ST1-infected plants produced the least number of pupae (213.0 ± 12.5) (Figure 6.3).

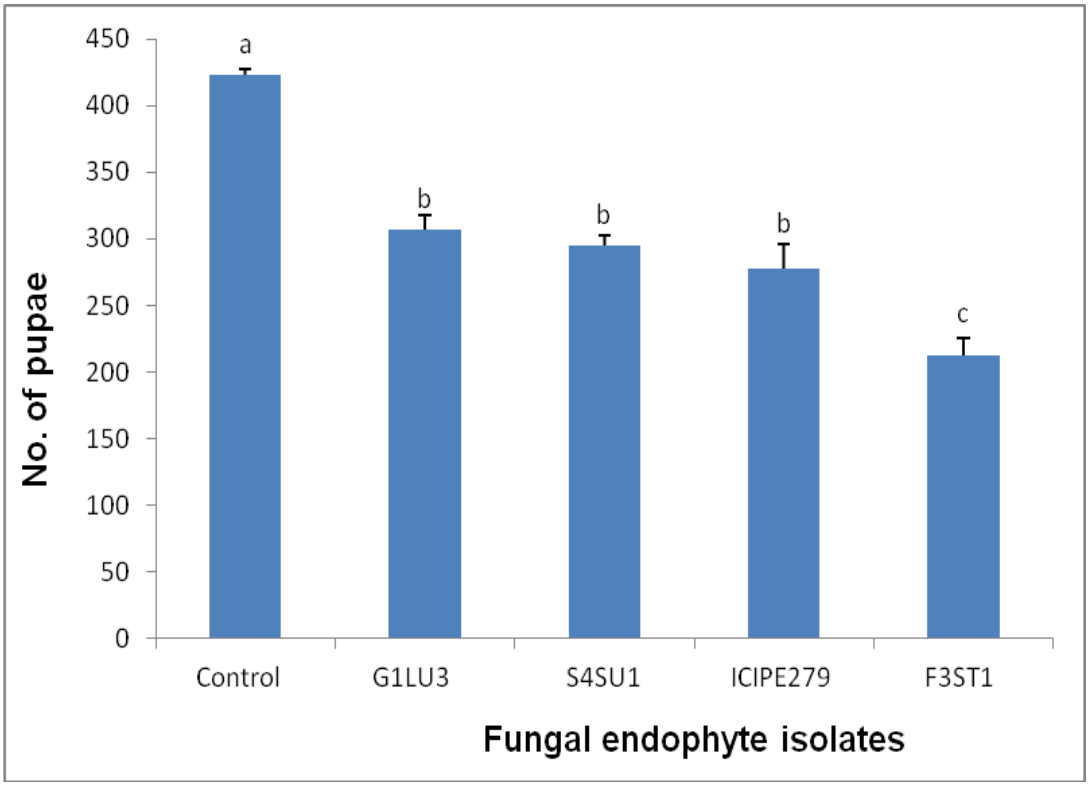

Figure 6.3: Effect of *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* (F3ST1) and infested with 2nd and 3rd instar of *Liriomyza huidobrensis* larvae on the number of pupae produced after *Diglyphus isaea* exposure. Bars denote means ± one standard error at 95% CI (p = 0.05).

6.2.2.4 Pupation after exposure of *Phaedrotoma scabriventris*

Fewer pupae were produced in endophytically-colonized plants than in the control (409.0 ± 6.0 pupae) (Figure 6.4). There were, however, significant differences (*F = 10. 29, df = 4, 9, p = 0.002*) between the fungal isolates and the control. No significant difference was observed among the fungal isolates. For example, the number of pupae produced in *B. bassiana* ICIPE 279 (304.0 ± 6.0) was significantly different from the control and *Hypocrea lixii* F3ST1-infected plants which produced the least number of pupae (217.0 ± 29.0) (Figure 6.4).

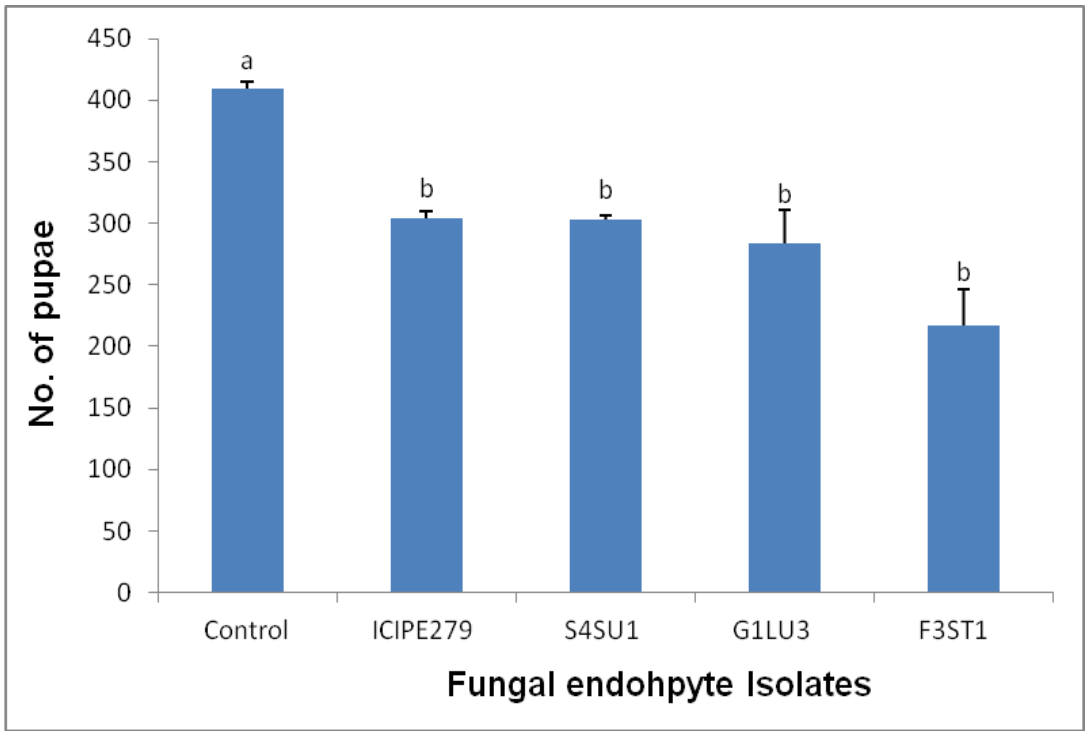

Figure 6.4: Effect of *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* (F3ST1) and infested with 2nd and 3rd instar of *Liriomyza huidobrensis* larvae on the number of pupae produced after *Phaedrotoma scabriventris* exposure. Bars denote means ± one standard error at 95% CI (p = 0.05).

6.2.2.5 Adult *Diglyphus isaea* emergence

Higher numbers of pupae emerged from control plants (339.5 ± 11.5) than from endophytically-colonized plants (Figure 6.5). There was a significant difference ($F = 12.24$, $df = 4, 9$, $p < 0.001$) in the numbers of pupal emergence in the fungal endophyte isolates as compared to the control (Figure 6.5). Fewer pupae emerged from plants endophytically-colonized by *H. lixii* F3ST1 (81.3 ± 19.8) as compared to the control (339.5 ± 11.5). However, no differences were observed among the four endophyte isolates (Figure 6.5).

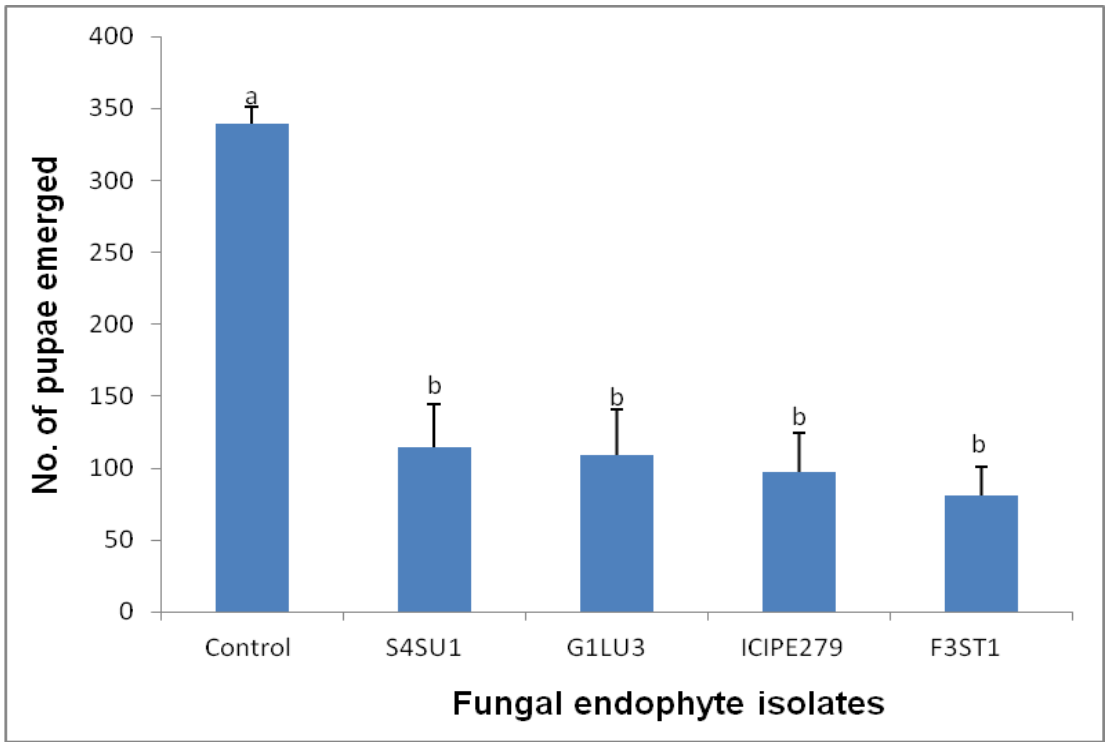

Figure 6.5: Effect of *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* (F3ST1) on adult emergence of *Liriomyza huidobrensis* and *Diglyphus isaea*. Bars denote means ± one standard error at 95% CI (p = 0.05).

6.2.2.6 Adult *Phaedrotoma scabriventris* emergence

The same trend was observed as in the case of *D. isaea*. Higher number of pupae emerged from control plants (299.5 ± 3.1) than from endophytically-colonized plants (Figure 6.6). There was a significant difference ($F = 56.52$, $df = 4, 9$, $p < 0.0001$) in the

numbers of pupae emerged in the fungal endophyte isolates as compared to the control (Figure 6.6). Fewer pupae emerged from plants endophytically-colonized by *H. lixii* F3ST1 (76.0 ± 3.5) compared to the control (299.0 ± 3.1). However, no differences were observed among the four endophyte isolates (Figure 6.6).

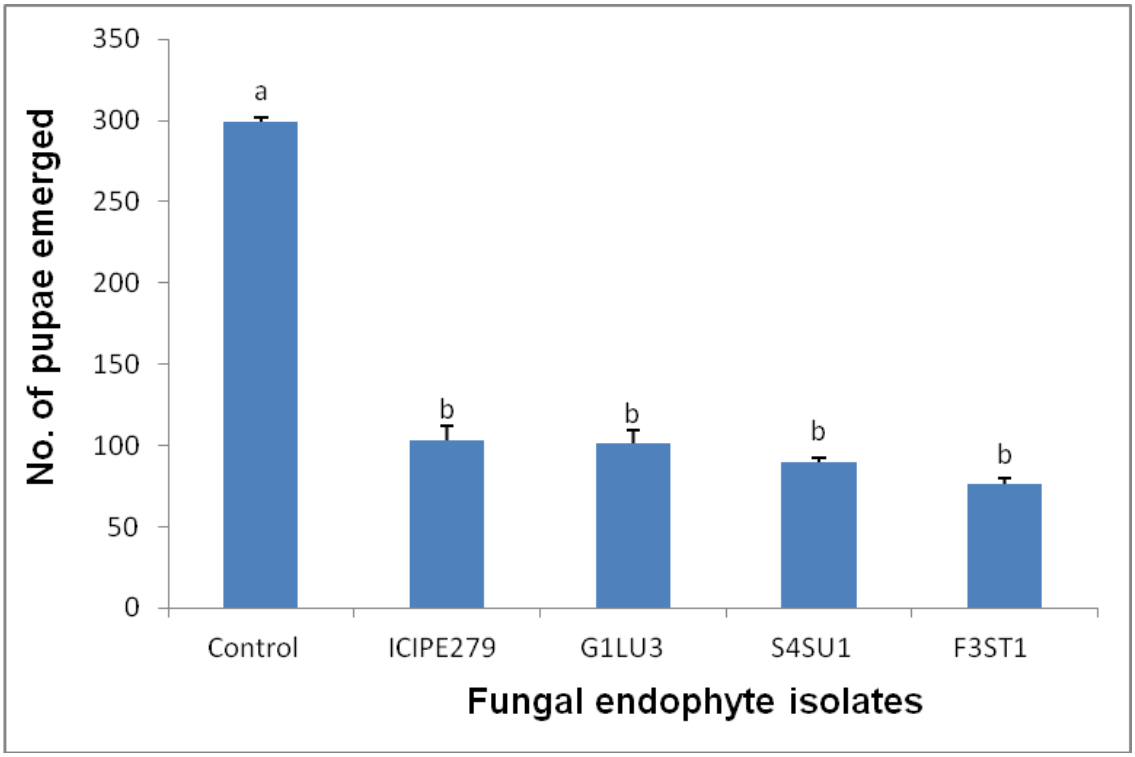

Figure 6.6: Effect of *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* (F3ST1) on adult emergence of *Liriomyza huidobrensis* and *Phaedrotoma scabriventris*. Bars denote means ± one standard error at 95% CI (p = 0.05).

6.2.2.7 Sex ratio

There was a significant difference in sex ratio between males and females among the endophyte isolate treatments for both *D. isaea* ($F = 0.75$, $df = 4, 9$, $p = 0.58$) and *P. scabriventris* ($F = 0.98$, $df = 4, 9$, $p = 0.47$).

In addition, no mycosis was observed among all the 1500 cadavers of *D. isaea* and *P. scabriventris* exposed to endophytically-colonized and infested *V. faba* plants.

6.2.3 Effects of endophytic fungal isolates on survival of *Diglyphus isaea* and *Phaedrotoma scabriventris* progenies

6.2.3.1 *Diglyphus isaea* progeny survival

The survival curves of the progeny from *D. isaea* whose parents were previously exposed to inoculated plants did not differ significantly among the treatments and the control (*proximate log rank test = 3.127, df = 4, p = 0.5367*) (Figure 6.7).

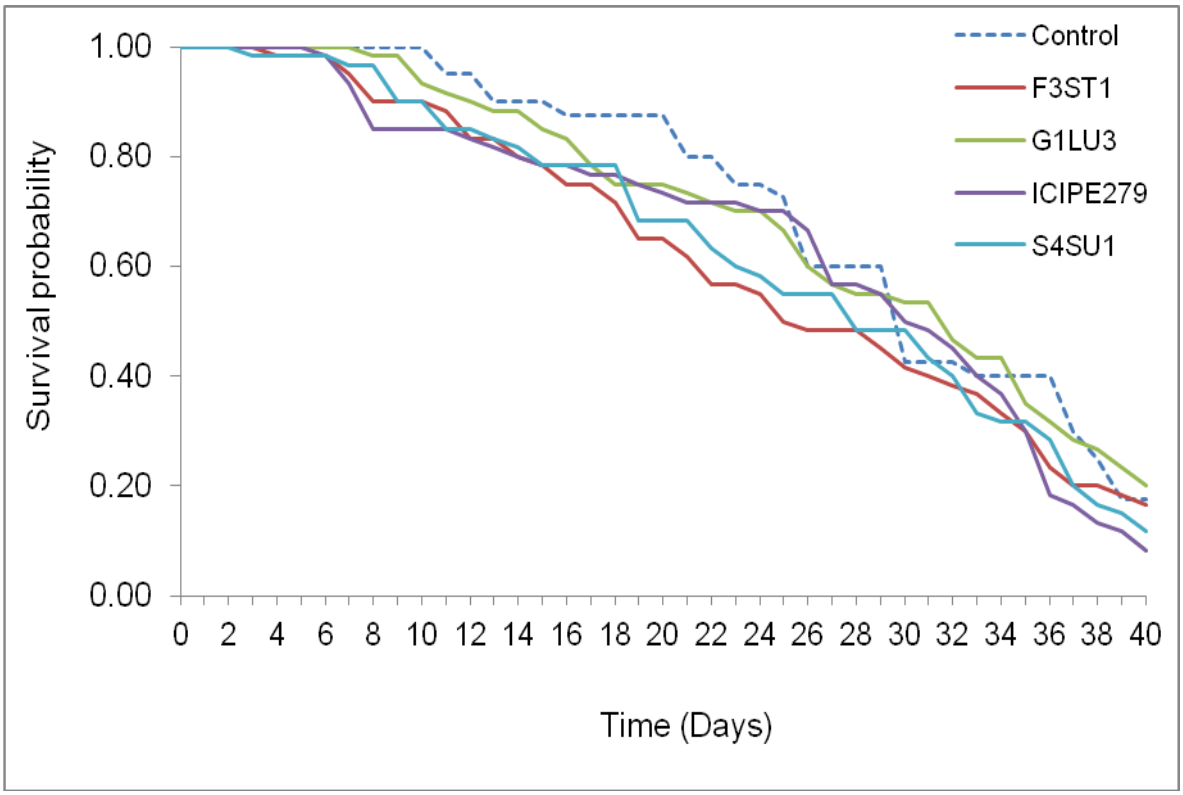

Figure 6.7: Progeny survival curves of *Diglyphus isaea* emerging from *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279) and *Hypocrea lixii* (F3ST1) and infested with 2nd and 3rd instar larvae of *Liriomyza huidobrensis*, 40 days post-emergence.

For instance, at 7 and 21 days post-emergence, the survival of *D. isaea* was above 95% and 70% respectively, in the control as well as in the endophyte treatments (Figure 6.7). At 40 days, the variations of the survival remained constant in the control and

treatments with uniform reduction to 17.5% in the control, 16.7% in *H. lixii* F3ST1, 20.0%, 11.7% and 8.3% in *B. bassiana* G1LU3, S4SU1 and ICIPE 279 respectively (Figure 6.7).

6.2.3.2 *Phaedrotoma scabriventris* progeny survival

The survival curves of the progeny from *P. scabriventris* whose parents were previously exposed to inoculated plants differed significantly among the treatments and the control (*proximate log rank test = 56.473, df = 4, p < 0.0001*) (Figure 6.8).

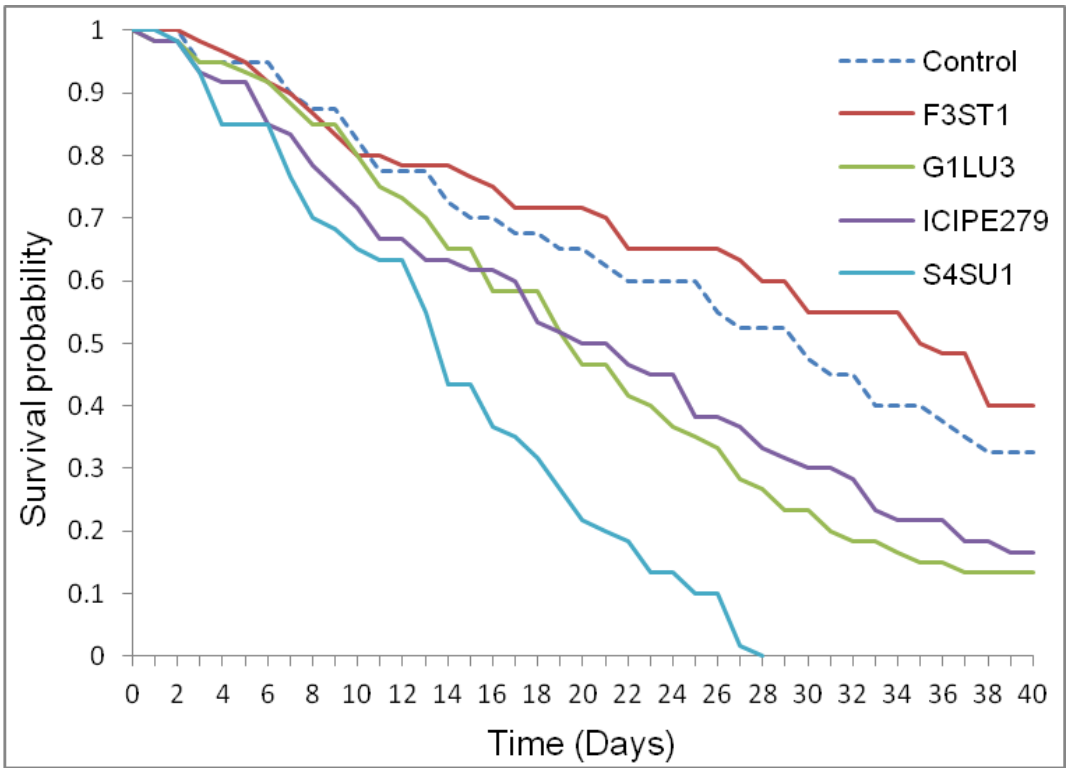

Figure 6.8: Progeny survival curves of *Phaedrotoma scabriventris* emerging from *Vicia faba* plants endophytically-colonized by different fungal isolates of *Beauveria bassiana* (S4SU1, G1LU3 and ICIPE279), and *Hypocrea lixii* (F3ST1) and infested with 2nd and 3rd instar larvae of *Liriomyza huidobrensis* 40 days post-emergence.

For example, at 7 days post-emergence, the survival of *P. scabriventris* was approximately at 90% in the control and other treatments, except in *B. bassiana* S4SU1 where it was 76.7%. At 28 days, no survival (0%) was observed in *B. bassiana* S4SU1

while 60%, 52.5%, 33.3% and 26.7% survival rates were observed in *H. lixii* F3ST1, control, *B. bassiana* ICIPE 279 and G1LU3 respectively (Figure 6.8). At 40 days, the survival rates were 40% and 32.5% in *H. lixii* F3ST1 and the control respectively, but were reduced to 16.7% and 13.3% in *B. bassiana* ICIPE 279 and G1LU3 respectively. There were greater survival rates of *P. scabriventris* in *H. lixii* F3ST1 than in the control from day 11 up to day 40 (Figure 6.8).

At a limited survival time of 40 days, the F1 progenies of *D. isaea* and *P. scabriventris*, there were no significant differences in the mean survival times between the control and the endophyte treatments for *D isaea* ($F = 0.34$, $df = 4, 9$, $p = 0.84$) and for *P. scabriventris* ($F = 1.42$, $df = 4, 9$, $p = 0.304$) respectively (Table 6.3). The endophytic fungi have not then affected the survival or longevity of the two parasitoids progenies, since 50% of the population of both parasitoids can survive more than 14 days (median survival time) (Table 6.3).

Table 6.3: Mean survival period of *Diglyphus isaea* and *Phaedrotoma scabriventris* F1 progeny, whose parents were exposed to *Liriomyza huidobrensis*-infested-*Vicia faba* plants colonized by the different endophytic fungal isolates

Fungal isolates species	**Mean survival time (Days) ± SE**	
	Diglyphus isaea	***Phaedrotoma scabriventris***
Beauveria bassiana ICIPE279	28.7 ± 2.8 [a]	20.5 ± 3.9 [a]
Beauveria bassiana G1LU3	29.5 ± 1.7 [a]	20.3 ± 3.1 [a]
Beauveria bassiana S4SU1	27.5 ± 2.0 [a]	14.5 ± 3.2 [a]
Hypocrea lixii F3ST1	27.0 ± 2.9 [a]	30.3 ± 8.7 [a]
Control	30.3 ± 2.8 [a]	28.0 ± 6.0 [a]

Means followed by the same letter in a column are not significantly different at 95% CI (p = 0.05).

6.3 Discussion

No negative effects were observed on the parasitism rates of *P. scabriventris* and *D. isaea*. However, the number of parasitoids emerged were fewer as compared to non-endophytically treated hosts. In *H. lixii* F3ST1 the least number of pupae was produced in both parasitoids. This result does not compromise the performance of the parasitoids but rather confirms the results reported by Akutse *et al*. (2013) where *H. lixii* F3ST1 reduced considerably the number of pupae as well as adult emergence of *L. huidobrensis*. Since the number of larvae which have reached pupation was reduced in the endophyte treatments, there may be also less which will be parasitized by the parasitoids. Additionally, no negative effects were observed on the survival of the exposed parent parasitoids as well as their respective progenies.

Tritrophic interactions involving parasitoids and caterpillars can take a variety of forms depending on the biological attributes of the insects' relationship and the type of plant upon which it occurs (Kennedy, 2003). Since solitary parasitoids often kill hosts earlier in larval development than gregarious ones, whose offspring need more resources for development (Senthamizhselvan and Muthukrishnan, 1989), *P. scabriventris* and *D. isaea* may escape the secondary chemical on which the larvae of *L. huidobrensis* fed on before been parasitized. As solitary parasitoids, *P. scabriventris* and *D. isaea* spend less time in the *Liriomyza huidobrensis* host larvae than poly-embrionic parasitoids. According to Bixby-Brosi and Potter (2011), *Copidosoma bakeri*, a polyembryonic wasp that develops from egg to adult within the host, would suffer greater negative fitness effects than would *Linnaemya comta* (Fallén) (Diptera: Tachinidae), a solitary, rapidly developing parasitoid, when their common host feeds on alkaloid-containing endophytic grass. The same authors reported that proportionately fewer parasitized cutworms yielded *C. bakeri* broods when the caterpillars consumed endophytically inoculated grass and tachinid, in contrast, did not appear to be affected by the presence of endophyte infection within its host plant. Similar results were obtained with the solitary parasitoids *D. isaea* and *P. scabriventris* in this study where their survival and parasitism rates were not affected by the endophytic fungal isolates.

Endophytically-inoculated plants can suppress certain leaf- and stem-feeding insect pests such as *L. huidobrensis* (Breen, 1994, Richmond *et al*., 2000, Akutse *et al*., 2013),

but the value of such an approach could be compromised if the endemic natural enemies of relatively endophyte-tolerant pests are disrupted. For example, *B. bassiana* S4SU1 reduced the survival time of *P. scabriventris* when inoculated to *V. faba* and infested with *L. huidobrensis* as compared to other isolates of *B. bassiana* (G1LU3 and ICIPE 279) and *H. lixii* F3ST1. Similar trends were observed where all the endophytic fungal isolates not only reduced *L. huidobrensis* parent and progeny survivals (Akutse *et al*., 2013) but also their emergence. However, although *B. bassiana* S4SU1 reduced the survival times of the exposed parasitoid parents, it had no effects on their progenies' survival times.

6.4. Conclusion

This study showed that no negative effects were observed on the parasitism rates of *P. scabriventris* and *D. isaea*. However, the number of parasitoids emerged were reduced as compared to non-endophytically treated hosts. In *H. lixii* F3ST1 the least number of pupae was produced in both parasitoids, but did not compromise the performance of the parasitoids in terms of parasitism but rather the reduction of the pest (*L. huidobrensis*) larvae. Additionally, no negative effects were observed on the survival of the exposed parent parasitoids as well as their respective progenies. This study highlights that tritrophic interactions between endophytic fungi, parasitoids, and endophytically-inoculated *V. faba* can differ depending on the particular species of parasitoids involved. The study also shows that the survival time of both parasitoids was not affected by *H. lixii* and *B. bassiana* ICIPE 279 and G1LU3. Since the survival and parasitism rates of both parasitoids were not affected, they may lay enough eggs during their life span to reduce the host pests' population. These endophytes can then be used in combination with the two parasitoids to control *Liriomyza* species. Endophyte effects on *P. scabriventris* and *D. isaea* warrant further study.

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CHAPTER 7: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.0 General discussion

The management of leafminers worldwide, and particularly in East Africa, has commonly relied on the routine use of synthetic chemical insecticides (Chandler, 1981, 1984; MacDonald, 1991; Gitonga *et al*., 2010). However, the indiscriminate and frequent use of these chemicals resulted in insecticide resistance of flies (Parella *et al*., 1984; Murphy and LaSalle, 1999), pollution of the environment as well as elimination of their natural enemies (Johnson *et al*., 1980; Murphy and LaSalle, 1999). Chemical control is also not very effective since flies usually escape insecticide applications due to their high mobility. Furthermore, *Liriomyza* larvae are inaccessible to many pesticides because they develop inside leaves and pupate in soil (Mujica and Kroschel, 2011). Biological control using parasitoids and entomopathogenic fungi is being considered as an alternative to leafminer management in East Africa (Migiro *et al*., 2010). The objectives of this study were therefore to investigate and assess the negative effects of fungal endophytes on *L. huidobrensis* following endophytic colonization of the host plants as well as the interactions between these endophytic fungi and *Liriomyza huidobrensis*, the introduced endoparasitoid *Phaedrotoma scabriventris* and the local ectoparasitoid *Diglyphus isaea* in order to improve biological control of these pests.

In the first experiment (Chapter 3), endophytic colonization of *V. faba* and *P. vulgaris* host plants by fungal pathogens was investigated. Colonization varied according to fungal isolate, host plant and different parts of the host plant. Isolates of *M. anisopliae* failed to colonize both *V. faba* and *P. vulgaris,* which contrasted with other studies (Akello, 2012; Sasan and Bidochka, 2012) who reported endophyte colonization of maize, Napier grass, beans, *Panicum virgatum* L. (Poaceae) by *M. anisopliae* and *M. robertsii,* respectively. There were differences in level of colonization of different parts of host plant among fungal isolates.

All the fungal isolates that endophytically-colonized *V. faba* were also pathogenic to *L. huidobrensis,* causing 100% mortality. *Hypocrea lixii* F3ST1 and *B. bassiana* G1LU3

significantly reduced the number of pupae produced by female *L. huidobrensis* and their emergence from pupal skins. Fungal endophytes also reduced survival of the *L. huidobrensis* adults, which corroborates the findings of Akello *et al.* (2008a) who reported reduction in larval survival of *C. sordidus* following colonization of banana by *B. bassiana*. Entomopathogens as endophytes can also slow down reproduction rate (longer time to reproduction and fertility), reduce feeding, growth and fecundity of insects (Gurulingappa *et al.,* 2010). Since more males emerged from endophyte-inoculated host plants than uninfected plants, this is an indication that most of the eggs laid by the females were not fertilized (Taylor and Yuval, 1999; Ward, 2000). In case where female insects experience limited resources or are in danger or under no-choice conditions (like in presence of metabolites produced by plants endophytically), they might simply avoid laying eggs or lay few eggs without fertilizing them (Charnov and Skinner, 1985). Additionally, the cryptic females choice might also be due to females response to plant growth and plant quality as described by Craig *et al.* (1992), where being male-biased could be a response to plant growth which is endophytically-colonized and progeny sex ratio varied in response to plant quality.

The life cycle of the F1-generation that emerged from *V. faba* plants inoculated with *H. lixii* F3ST1, *B. bassiana* S4SU1 and *T. asperellum* M2RT4 was shorter than one of the other isolates and control. Vega *et al.* (2008) reported similar effects of an endophytic *B. bassiana* SPCL 03047 on adult *H. hampei* with an average survival period of 4.8 ± 0.2 days compared to 15.0 ± 0.6 days for uninoculated plants.

The fact that no mycosed insects were recorded suggests that adult mortality of *L. huidobrensis* could be as a result of feeding deterrence or antibiosis (Lewis and Cossentine, 1986; Lewis and Bing, 1991; Bing and Lewis, 1991; Bing and Lewis, 1992a, b; Lewis *et al.,* 1996; Cherry *et al.,* 2004; Vega *et al.,* 2008). The mechanism by which endophytic fungi interact with insects is still unclear but one can speculate on production of metabolites/antibiosis (Vega *et al.,* 2008). Lewis and Bing, (1991) and Bing and Lewis, (1991, 1992a, b) suggested that the reduced tunneling of *Ostrinia nubilalis* (Lepidoptera: Crambidae) following endophyte colonization of maize by *B. bassiana* was due to the presence of fungal metabolites that cause feeding deterrence or antibiosis. This might explain the absence of *B. bassiana* infection (mycosis) within *O.*

nubilalis individuals that fed on endophytically-infected plants. Studies by Cherry *et al.* (2004) on *Sesamia calamistis* (Lepidoptera: Noctuidae) also supported the feeding deterrence/antibiosis hypothesis since larvae feeding on plants injected with *B. bassiana* were smaller than those in the control plants.

The simultaneous use of *P. scabriventris* and *D. isaea* in the management of *L. huidobrensis* resulted in a higher parasitism rate compared to each of them used separately (Chapter 4). The parasitism rate of *D. isaea* and *P. scabriventris* obtained in the present study was similar to the one reported by Boot *et al.* (1992), who reported 30% parasitism by *D. isaea* in *L. bryoniae*. *Phaedrotoma scabriventris* performed better than *D. isaea* in terms of specific parasitism, showing that it is a superior parasitoid compared to *D. isaea* and therefore its release in the horticultural production systems will fill the existing gap of lower parasitism rates reported in Kenya by Chabi-Olaye *et al*. (2008) and substantially contribute to the suppression of *Liriomyza* species as well as enhancing horticulture production in Kenya. On the other hand, *D. isaea* cause a considerably higher feeding and stinging mortality to *L. huidobrensis* as compared to *P. scabriventris,* despite that it has a lower parasitism rate. This important additional mortality through parasitoid feeding-stinging behavior needs to be accounted for when evaluating their performance. In addition, *P. scabriventris* did not have any negative effect on the performance of *D. isaea*. There was no multiparasitism, hyperparasitism or superparasitism among and between *D. isaea* and *P. scabriventris*. Although *P. scabriventris* had no effect on *D. isaea*, the presence of *D. isaea* reduced slightly the performance of *P. scabriventris* in terms of parasitism. However, both parasitoids used together led to a higher total parasitism. This is an indication that no harmful effect under field conditions is expected after release of *P. scabriventris* and the association of both parasitoids will result into a more pronounced reduction of the leafminer flies in East Africa.

Successful co-existence of *P. scabriventris* and various *Diglyphus* species was reported by Mujica and Kroschel (2007), Valladares *et al*. (1999) and Kroschel (2008). According to Minkenberg (1989), *D. isaea* occurs widely as an ectoparasitoid of leafmining dipteran larvae on herbaceous plants in Europe, Northern Africa and Japan. Competitions between leafminer parasitoids are not known and hyperparasitism has

never been observed in any leafminer fly field samples taken over many years (Murphy and LaSalle, 1999; Mujica and Cisneros, 1997; Mujica and Kroschel, 2007; Chabi-Olaye *et al*., 2008). For instance, in Peru, the leafminer fly complex has more than 60 different parasitoid species which co-exist (Mujica and Kroschel, 2007). It is therefore expected that, after the release of the introduced parasitoid *P. scabriventris*, higher parasitism rates of leafminer flies will be obtained and hence better biological control achieved.

In addition to the mortality induced by *P. scabriventris* and *D. isaea* through oviposition (parasitism rate) discussed above, another important additional mortality factor was parasitoid feeding-stinging which need to be accounted for when evaluating their performance. Minkenberg (1989) and Anonymous (2007) also reported that the females of *D. isaea* not only lay eggs on larvae, but also host feed on them (host feeding), sting and introduce toxin into larvae (host stinging) before laying eggs. Host feeding-stinging by parasitoids therefore considerably reduced the offspring of the *L. huidobrensis* population beyond the simple parasitism rate.

Morphological identification of fungal isolates obtained during seeds assessment (bioprospecting) was confirmed by molecular techniques (Chapter 5). Results from the two gene regions utilized yielded similar results, confirming that the morphological identification of the isolates are in line with the molecular identification and indeed the endophytes belong to the four groups of *Metarhizium*, *Beauveria*, *Epacris* and *Phanerochaete .* Both the two ITS 5 & 4 and AB28 & TW81 gene regions linked the CR, MR and MF and the *icipe* standards to the *Metarhizium anisopliae* spp. though they occupied different branches from the standards, while the BF linked to the *Beauveria* spp as expected and also branched differently from the *icipe Beauveria* standards. Akello (2012) has also identified and reported S4ST7 isolate as *M. anisopliae* using IGS and ITS primers for molecular characterization. This confirmed our result with S4ST7 using ITS 5 & 4 and AB28 & TW81 regions and therefore suggesting alternate primers that can be used in fungal characterization.

The presence of these endophytes in *V. faba* and *P. vulgaris* seeds shows a high chance that the seedlings of these seeds may be colonized by the same fungal endophytes either naturally or artificially through inoculation. The *Epacris microphylla*

which was identified as an endophyte in this study was also identified as an endophytic fungus associated with the Chinese traditional medicinal plant *Achyranthes bidentata* Blume (Amaranthaceae) (Bing-Da *et al*. 2013). Since these same genera were found to be effective endophytic fungi against *L. huidobrensis* (Chapter 3), and exist naturally in the seeds, they may not therefore have any negative effects on non-target species.

No negative effects were observed on the parasitism rates of *P. scabriventris* and *D. isaea* (Chapter 6). However, the number of parasitoids emerged were few as compared to non-endophytcally treated hosts. In *H. lixii* F3ST1 the least number of pupae was produced in both parasitoids. This result does not compromise the performance of the parasitoids but rather confirms the results reported by Akutse *et al*. (2013) where *H. lixii* F3ST1 reduced considerably the number of pupae as well as adult emergence of *L. huidobrensis*. Since the number of larvae which reached the pupation stage was few in the endophyte treatments, there may be also few which will be parasitized for parasitoid development. Additionally, no negative effects were observed on the survival of the exposed parent parasitoids as well as their respective progenies. However, the survival of *P. scabriventris* progeny was reduced to 28 days when the hosts were treated with *B. bassiana* S4SU1.

7.1 Conclusions

Of the ten fungal isolates screened, eight of them colonized both *V. faba* and *P. vulgaris*, suggesting that they could also be potential endophytes on other Fabaceae plants, although this would need to be confirmed. Further studies are warranted to identify the range of *Liriomyza* spp. host plants in which endophytic properties could be expressed by these isolates. The two *M. anisopliae* isolates did not colonize any of the two host plant species. *Hypocrea lixii* F3ST1 and *B. bassiana* (ICIPE279, S4SU1 and G1LU3) were the most pathogenic isolates against *L. huidobrensis* in causing mortality, reducing adult survival, number of pupae, progeny survival and adult emergence. The endophycally-colonized host plant had negative effects on the host insect; further research is therefore warranted in elucidating the mode of action and their use in the management of *L. huidobrensis.* It was also observed that endophycally-colonized *V. faba* had repellent effects on the red spider mites, *Tetranychus evansi* and *T. urticae*

(Annex 1). Studies are still underway to investigate the effects of these endophytes on red spider mites.

Phaedrotoma scabriventris performed better than *D. isaea* in terms of parasitism rate and represents a superior parasitoid compared to *D. isaea.* Simultaneous use of both parasitoids resulted in higher parasitism and mortalities due to feeding and stinging. No multiparasitism, superparasitism or hyperparasitism were observed within and among the two parasitoids. Field release of *P. scabriventris* will contribute to the currently low levels of parasitism in Kenya. Additionally, *P. scabriventris* has no negative effect on the parasitism rate or feeding and stinging mortalities caused by *D. isaea.*

In Chapter 5, *Beauveria bassiana* isolate BF, *M. anisopliae* isolates MF, MR, *Phanerochaete chrysosporium* ZF and *Epacris microphylla* ZRA were found as natural seed fungal endophytes while *M. anisopliae* CR was identified as a *P. vulgaris* root endophyte. They were clearly different from the standards *B. bassiana* ICIPE279 and *M. anisopliae* ICIPE279 and S4ST7 despite clustering into the group. The advantages of this characterization is that it will assist the scientific community not only in awareness about seed endophytes but also to see if the presence of these fungi will (i) inhibit/suppress the endophytes growth/colonization which will be artificially inoculated (specially through seed inoculation) inside the host plants for pest control in general and *Liriomyza* leafminer species particularly, (ii) have any additive, symbiotic or synergic effects in the management of *Liriomyza* leafminers and other pests, (iii) test also pathogenicity and virulence of these seed endophytes on different sucking arthropods *in vitro* and *in vivo*, (iv) assess the quality of the seeds in presence of these endophytes, (v) assess the nutritional value of the seeds containing the endophytes and finally (vi) assess the effects of the endophytes on stored products insects when storing seeds derived from endophytically inoculated plants.

No negative effects were observed on the parasitism rates of *P. scabriventris* and *D. isaea* (Chapter 6). Additionally, the endophytic fungal treated-hosts did not effect the survival of the exposed parent parasitoids as well as their respective progenies. However, the survival of *P. scabriventris* progeny was reduced to 28 days when the hosts were treated with *B. bassiana* S4SU1.

7.2 Recommendations

1) Further studies are warranted to identify the range of *Liriomyza* spp. horticultural host plants in which endophytic properties could be expressed by fungal endophyte isolates.

2) Since repellent effect of endophytically-colonized plant was observed on red spider mites, there is the need to evaluate the host range, including post-harvest insect pests, of these fungal endophytes.

3) The effect of endophytically-colonized plant on plant diseases also requires particular attention as it has been reported elsewhere.

4) Since *Hypocrea lixii* F3ST1 and *B. bassiana* (ICIPE279, S4SU1 and G1LU3) were the most pathogenic isolates against *L. huidobrensis* in causing mortality, reducing adult survival, number of pupae, progeny survival and adult emergence at the laboratory, further studies are warranted to test them at the field level.

5) It is also important to screen isolates of *Hypocrea lixii* and *B. bassiana* for pathogenicity on *L. huidobrensis*. The identification of more pathogenic isolates would improve mortality control.

6) The underlying mechanism by which endophytic fungal pathogens affect insect pests warrant further research.

7) *Phaedrotoma scabriventris* and *D. isaea* co-exist and have complementary parasitism effects. No harmful effect is expected on *D. isaea* after release of *P. scabriventris* in East Africa. The association of both parasitoids will result in increased mortality and parasitism rates and hence improved biological control of leafminers.

8) Further studies are warranted to assess the vertical transmission of the seeds endophytes to their seedlings and their effects on the artificial seed inoculation technique.

9) The pathogenicity and virulence of these seeds endophytes on different sucking arthropods such as mites also need further study. Seed quality and nutritional value of seeds containing endophytes needs to be determined. The effects of these endophytes on storage pests when storing seeds derived from endophytically inoculated plants also deserves further sudy.

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Annexes

Annex 1: Effects of endophytically inoculated-*Vicia faba* plants on red spider mites (RSM) in the green house at *icipe*.

A) Red spider mites feeding on the control plants of *Vicia faba* and making webs.

A) Damage caused by RSM to *Vicia faba* plant in the green house (left)

B) Healthy/non-attacked inoculated *Vicia faba* plants at the green house (right)

Annex 2: Consolidated sequences of the isolated fungal endophytes using ITS 5 & 4 primers

>S4ST7: *Metarhizium anisopliae*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCC
CTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGAC
CCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG
TCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCG
TCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA
CTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATA
GTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAA

>CR: *Metarhizium anisopliae*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCC
CTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGAC
CCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG
TCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCG
TCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA
CTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATA
GTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAA

>ICIPE 279: *Beauveria bassiana*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCT
TCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGAC
TGGACCAGCGGCCCGCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAAT
ACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCT

GGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCAT
GCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGG
GGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCG
ACCTCTGCGCAGTAATACAGCTCGCACCGGGACCCCGACGCGGCCACGCCGTAA
AACACCCAACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTA
A

>MF: ***Metarhizium anisopliae***

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCC
CTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGAC
CCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG
TCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCG
TCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA
CTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATA
GTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAA

>MR: ***Metarhizium anisopliae***

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCC
CTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGAC
CCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG
TCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCG
TCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA
CTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATA
GTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAA

>ZF: *Phanerochaete chrysosporium*

TTTCCGTAGGTGAACCTGCGGAAGGATCATTAACGAGTAACTGAACAGGTTGTAGC
TGGCCTTTCGGGGCATGTGCACACCTGGCTCATCCACTCTTCAACCTCTGTGCACT
TGTTGTAGGTCGGTAGAAGAGCGAGCATCTAATGCTTGCTTGGAAGCCTTCCTATG
TTTTACTACAAACGCTTCAGTTTAAGAATGTTTACCTGCGTATAACGCATTTATATAC
AACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCCTGGTATTCCGGGGAGCATGCCTGTTTGAGTGTCATGGTATTCTCAACCT
TCATAACTTTTGTTATCGAAGGCTTGGACTTGGAGGTTGTGCTGGCTTCTAGTTAAG
TCGGCTCCTCTTAAATGTATTAGCGTGAGTGTAACGGATCGCTTCGGTGTGATAAT
TATCTGCGCCGTGGTCGTGAAGTAACATAAGCTTGCGCTTCTAACCGTCCTTCAGT
TGGACAATTACTTTGACATCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTA
A

>ZRA: *Epacris microphylla*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACAGAGTTGCAAAACTCCCAAACC
ATTTGTTTATCTACCTGTTTCGTTGCTTCGGCAGGCGGCTCTTCGGGGCCTTTGCT
ACCTGTAGTGCCTGCCGGAGTATCCAAAACTCTTGTTATTTTTGTTCTGTCTGAGTA
AACTTTTAATAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG
AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT
CTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTC
ATTTCAACCCTCAAGCTCTCTTGCTTGGTGTTGGGGCTTCTGCGGCTTCGGCCGCA
GGCCCTGAAAAACAGTGGCGGGCTCGCTATAACTCCGAGCGTAGTAATCTCTCTC
GCTTTGGAAGTGTGGCGGTTCCCGGCCGTTAAACCCCCCAATTTCTGAAATGTTGA
CCTCGGATCAGGTAGGAATACCCGCTGAACTTAA

>BF: *Beauveria bassiana*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCT
TCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGAC
TGGACCGGCGGCCCGCCGGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAAT
ACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCT
GGCATCGATGAAGAACGCAGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCAT

GCCTGTTCGAGCGTCATTTCAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGG
GGACCGGCAGCACACCGCCGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCG
ACCTCTGCGCAGTAATACAGCTCGCACCGGGACCCCGACGCGGCCACGCCGTAA
AACACCCAACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTA
A

>ICIPE 30: *Metarhizium anisopliae*

TCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTATCCAACTCCCAACCC
CTGTGAATTATACCTTTAATTGTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGAC
CCAAACCTTCTGAATTTTTTAATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG
CGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAG
TCCCCTGTGGGACTTGGTGTTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCG
TCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCA
CTCGCAACAGGAGCCCGGCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATA
GTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAA

Annex 3: Consolidated sequences of the isolated fungal endophytes using AB28 & TW81 primers

>BF: *Beauveria bassiana*

GGAGGGATCATTACCGAGTTTTCAACTCCCTAACCCTTCTGTGAACCTACCTATCG
TTGCTTCGGCGGACTCGCCCCAGCCCGGACGCGGACTGGACCGGCGGCCCGCC
GGGGACCTCAAACTCTTGTATTCCAGCATCTTCTGAATACGCCGCAAGGCAAAACA
AATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGC
AGCGAAACGCGATAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT
CAACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACACCGC
CGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAGTAATACA
GCTCGCACCGGGACCCCGACGCGGCCACGCCGTAAAACACCCAACTTCTGAACG
TTGACCTCGAATCAGGTAGGACT

>MR: *Metarhizium anisopliae*

GGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATT
GTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTA
ATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGGACTTGGTG
TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCG
GTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCG
GCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGT
AGGACT

>ZRA: *Epacris microphylla*

GGAGGGATCATTACAGAGTTGCAAAACTCCCAAACCATTTGTTTATCTACCTGTTTC
GTTGCTTCGGCAGGCGGCTCTTCGGGGCCTTTGCTACCTGTAGTGCCTGCCGGAG
TATCCAAAACTCTTGTTATTTTTGTTCTGTCTGAGTAAACTTTTAATAGTTAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC

CATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTC
TTGCTTGGTGTTGGGGCTTCTGCGGCTTCGGCCGCAGGCCCTGAAAAACAGTGGC
GGGCTCGCTATAACTCCGAGCGTAGTAATCTCTCTCGCTTTGGAAGTGTGGCGGTT
CCCGGCCGTTAAACCCCCCAATTTCTGAAATGTTGACCTCGGATCAGGTAGGAAT

>CR: *Metarhizium anisopliae*

GGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATT
GTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTA
ATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGGACTTGGTG
TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCG
GTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCG
GCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGT
AGGACT

>ICIPE: 30 *Metarhizium anisopliae*

GGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATT
GTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTA
ATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGGACTTGGTG
TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCG
GTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCG
GCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGT
AGGACT

>MF: *Metarhizium anisopliae*

GGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATT
GTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTA
ATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTC

TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGGACTTGGTG
TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCG
GTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCG
GCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGT
AGGACT

>ICIPE: 279 *Beauveria bassiana*

TAACCTTTTAAGAATAGTATGAGGTACGTTCAGAAGTTGGGTGTTTTACGGCGTGG
CCGCGTCGGGGTCCCGGTGCGAGCTGTATTACTGCGCAGAGGTCGCCGCGGACG
GGCCGCCACTCCATTTCAGGGCCGGCGGTGTGCTGCCGGTCCCCAACGCCGACC
TCCCCAAGGGGAGGTCGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCCAG
AATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGGATTCTGCAA
TTCACATTACTTATCGCGTTTCGCTGCGTTCTTCATCGATGCCAAAGCCAAGAAATC
CGTTGTTGAAAGTTTTGATTCATTTGTTTTGCCTTGCGGCGTATTCAGAAGATGCTG
GACATACAAGGAGTTTGAGGTCCCCGGCGGGCCGCTGGTCCAGTCCGCGTCGCG
GGCTGGGGCGAGTCCGCCGAAGCAACGATAGGTAGGTTCACAGAAGGGTTAGGG
AGTTGAAAACTCGGTAATGATCCCTCCGCAGGTTCACCTACGGAAACAAAAGGGC
CT

>S4ST7: *Metarhizium anisopliae*

GGAGGGATCATTACCGAGTTATCCAACTCCCAACCCCTGTGAATTATACCTTTAATT
GTTGCTTCGGCGGGACTTCGCGCCCGCCGGGGACCCAAACCTTCTGAATTTTTTA
ATAAGTATCTTCTGAGTGGTTAAAAAAAATGAATCAAAACTTTCAACAACGGATCTC
TTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGTCAGTATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTACGCCCCTCAAGTCCCCTGTGGGACTTGGTG
TTGGGGATCGGCGAGGCTGGTTTTCCAGCACAGCCGTCCCTTAAATTAATTGGCG
GTCTCGCCGTGGCCCTCCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCG
GCGCGGTCCACTGCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGT
AGGACT

>ZF: *Phanerochaete chrysosporium*

GGAAGGATCATTAACGAGTAACTGAACAGGTTGTAGCTGGCCTTTCGGGGCATGT
GCACACCTGGCTCATCCACTCTTCAACCTCTGTGCACTTGTTGTAGGTCGGTAGAA
GAGCGAGCATCTAATGCTTGCTTGGAAGCCTTCCTATGTTTTACTACAAACGCTTCA
GTTTAAGAATGTTTACCTGCGTATAACGCATTTATATACAACTTTCAGCAACGGATC
TCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCCTGGTATTCCG
GGGAGCATGCCTGTTTGAGTGTCATGGTATTCTCAACCTTCATAACTTTTGTTATCG
AAGGCTTGGACTTGGAGGTTGTGCTGGCTTCTAGTTAAGTCGGCTCCTCTTAAATG
TATTAGCGTGAGTGTAACGGATCGCTTCGGTGTGATAATTATCTGCGCCGTGGCG
GTGAAGTAACATAAGCTTGCGCTTCTAACCGTCCTTCAGTTGGACAATTACTTTGAC
ATCTGACCTCAAATCAGGTAGGACT