Ecology and Biocontrol options of the Leaf Miner, *Liriomyza huidobrensis*, on Potatoes in the Western Cape province

by

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DECLARATION

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SUMMARY

The potato leaf miner, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae), is a devastating pest of potatoes, worldwide. However, little knowledge of the biology and ecology of this pest in South Africa exists, with both factors being cardinal elements in implementing an effective integrated pest management (IPM) programme. Control of the pest relies on the extensive use of chemical insecticides. Concerns regarding insect resistance, human safety and environmental impacts have, however, encouraged the development and use of alternative environmental control strategies, including the use of entomopathogenic nematodes (EPNs) and fungi (EPF). The present study focuses on the population dynamics of *L. huidobrensis* during the winter and summer period, a parasitoid survey and biological control using EPNs and EPF. The study showed differences in adult leaf miner populations in both the potato fields and the natural vegetation for both the winter and summer monitoring periods. The abundance and population densities of leaf miners increased during summer, while being very low during the winter monitoring period. The observed trend was that the leaf miner populations increased during crop development, with damage regularly being spotted on the potato leaves two weeks after plant emergence. Five parasitoid species associated with *L. huidobrensis* were identified during the monitoring periods, with *Diglyphus isaea* being the most abundant species. After assessing the occurrence of entomopathogens in the soil samples collected from the potato fields, one EPN species *Heterorhabditis zealandica*, one insect-associated nematode *Acrobeloides* sp. and two EPF species, *Metarhizium robertsii* and *Metarhizium majus* were isolated from the 16 surveyed potato fields. Four EPN species were screened for pathogenicity against the larval and pupal stages using different concentrations of infective juveniles (IJs) in the laboratory, which included *Heterorhabditis bacteriophora*, *Heterorhabditis baujardi* and *Steinernema yirgalemense* and which were obtained from the collection of the Department of Conservation Ecology and Entomology, Stellenbosch University, with the fourth species *Heterorhabditis zealandica* being isolated from the potato fields. The pathogenicity results of all the EPNs at 200 IJs/50 µl of distilled water solution indicated insect mortality of > 40 % for both the leaf mining larvae and the pupae. However, pupae and larvae trials indicated that the best performing treatment was found to be *H. baujardi* with more than 57 % of pupae mortality and > 71 % larvae mortality. The findings, thus, indicate the EPNs potential for adoption as part of an IPM strategy. On the other hand, two different species of EPF, namely *Metarhizium robertsii* and *Beauveria bassiana*, were tested for pathogenicity against the pupae of the potato leaf miner. The EPF were able to cause 67 % mortality. The study also screened five different isolates of *M. robertsii* found during the monitoring period, with the results obtained

indicating very high pupae mortality of more than 80 % of pupae. The study provided a positive forecast for the management of potentially damaging populations using entomopathogens as biocontrol agents against *L. huidobrensis*, in potato cultivation, in South Africa. This study was a laboratory assessment and thus a first step towards further development of a management strategy.

OPSOMMING

Die aartappelblaarmyner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), is 'n ernstige pes van aartappels wêreldwyd. Daar is egter min bekend oor die biologie en ekologie van hierdie pes in Suid-Afrika en beide hierdie faktore is van kardinale belang vir die implementering van 'n effektiewe geïntegreerde pesbeheerstelsel (IPM). Die bestryding van die *L. huidobrensis* is afhanklik die gebruik van chemiese insekdoders. Insekweerstand, menslike veiligheid en die effek op die omgewing het egter die ontwikkeling en gebruik van alternatiewe omgewingsbeheerstrategieë aangemoedig, insluitend die gebruik van entomopatogeniese nematodes (EPNs) en entomopatogeniese fungi (EPF). Hierdie studie fokus op die bevolkingsdinamika van *L. huidobrensis* gedurende die winter en somer, 'n parasitoïed opname en biologiese beheer met behulp van EPNs en EPF. Die studie het verskille getoon in die populasies van blaarmyners by volwassenes, in beide die aartappellande en die natuurlike plantegroei gedurende die winter- en somer moniterings periodes. Die bevolkingsgetalle en digtheid van blaarmyners het gedurende die somer toegeneem, terwyl dit gedurende die winter moniterings periode baie laag was. Die waargenome tendens was dat die blaarmynerpopulasies toegeneem het tydens gewas ontwikkeling, met skade wat gereeld op die aartappelblare opgemerk is twee weke na die opkoms van plante. Vyf parasitoïed spesies, wat verband hou met *L. Huidobrensis*, is tydens die moniteringstydperke geïdentifiseer, met *Diglyphus isaea* wat die mees algemeen waargeneem is. Nadat die voorkoms van entomopatogene in grondmonsters wat van 16 aartappellande versamel is, vasgestel is, is die volgende spesies geïsoleer: een EPN spesie naamlik, *Heterorhabditis zealandica*, een insek geassosieerde nematode, *Acrobeloides* sp. en twee EPF spesies, *Metarhizium robertsii* en *Metarhizium majus*. Vier EPN spesies is in die laboratorium vir patogenisiteit teen die larwale- en papiestadia getoets deur verskillende konsentrasies van infektiewe larwes (IJ's) te gebruik. Hierdie spesies sluit in: *Heterorhabditis bacteriophora*, *Heterorhabditis baujardi* en *Steinernema yirgalemense* wat verkry is uit die versameling van die Departement van Bewaringsekologie en Entomologie, Universiteit Stellenbosch. Die vierde spesie, *H. zealandica* is van die aartappellande geïsoleer. Die patogenisiteitsresultate van al die EPNs met 'n konsentrasie van 200 IJs / 50 µl gedistilleerde water dui op insekmortaliteit van >40% vir beide die blaarmyner larwes en die papies. Papie- en larfproewe het egter aangedui dat *H. baujardi* die beste behandeling is met meer as 57% van die papiesterftes en >71% larvale mortaliteit. Die bevindinge dui dus op die potensiaal vir aanvaarding van EPNs as deel van 'n IPM-strategie. Twee verskillende soorte EPF, naamlik *Metarhizium robertsii* en *Beauveria bassiana*, is ook getoets vir hul vatbaarheid teen die papies van die aartappelblaarmyner. Die EPF kon tot 67% mortaliteit veroorsaak. Vyf verskillende isolate van *M. robertsii*, wat gedurende die moniteringstydperk gevind is, getoets en

die resultate dui op 'n baie hoë papiemortaliteit van meer as 80%. Die studie dui daarop dat entomopatogene as biobeheermiddels die potensiaal het om baie suksesvol te wees vir die beheer van skadelike populasies van *L. huidobrensis* in aartappelverbouing in Suid-Afrika. Hierdie studie was 'n laboratorium assessering, en dus 'n eerste stap in die verdere ontwikkeling van 'n bestuurstrategie vir *L. huidobrensis*.

This thesis is dedicated to my young brother (Joseph Wesley Mugala) and my Cousin (Mr. Richard Musukwa) who has played a father figure ever since my Father passed on. Thank you for believing, supporting, and trusting me.

BIOGRAPHICAL SKETCH

Thabu Mugala earned her Bachelor of Science degree in Agronomic engineering at EARTH University in Costa Rica in the year 2018. Her final year's project evaluated the susceptibility of *Spodoptera frugiperda* (Fall army worm) to different strains of fungi and bacteria. Her Master of Science degree in Entomology, is focused on the biological control of the leaf-mining insect pest that affects potato leaves in South Africa.

Her interests include food security and hunger reduction and as such aims to raise awareness on current global challenges that contribute to food insecurity. Her motivation is to contribute significantly to the agriculture industry through the integration of local farmers.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal of the Entomological Society of Southern Africa (*African Entomology*).

Chapter 1 **Literature review**

Liriomyza huidobrensis (Diptera: Agromyzidae) on potatoes in South Africa, with special reference to biological control entomopathogens and parasitoids

Chapter 2

Occurrence and Distribution of the Potato Leaf Miner, *Liriomyza huidobrensis* (Diptera: Agromyzidae) and Parasitoids in Potato and Natural Vegetation of South Africa

Chapter 3

Isolation of Entomopathogenic Nematodes and Fungi for the Control of the Potato Leaf miner, *Liriomyza huidobrensis* (Diptera: Agromyzidae)

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LITERATURE REVIEW

Liriomyza huidobrensis (Diptera: Agromyzidae) on potatoes in South Africa, with special reference to biological control entomopathogens and parasitoids

ABSTRACT

Although agriculture is crucial to economic growth in South Africa, the industry faces several challenges, including the effects of pests and diseases that contribute to crop loss. Such losses severely affect the maintenance of food security. Therefore, a major concern is finding effective, environmental-friendly control measures for insect pests. Potatoes are among the four most widely consumed vegetable crops worldwide. However, a potato crop can be infested by various pests, like the devastating leaf miner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae). The leaf miner species has, since the early 2000s, become the most important pest of vegetable crops nationwide. The species is highly invasive, causing up to 70 % damage of solanaceous crops. The damage that is caused by the leaf miner is direct, resulting from the female flies feeding on the leaf mesophyll during oviposition, and the larvae mining the leaves. Indirect damage is induced through pathogens entering through perforations that act as vectors of plant diseases. Such biocontrol agents as entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPF) and parasitoids have shown biological control potential against *L. huidobrensis*. This review investigates the biology and morphological identification of *L. huidobrensis*, its host range in the Western Cape, and the potential of associated biocontrol agents, like EPNs, EPF and parasitoids, as future control options.

Key words: biocontrol agents, entomopathogenic nematodes, entomopathogenic fungi, potato leaf miner, parasitoids

INTRODUCTION

Liriomyza huidobrensis (Blanchard) (Diptera: Agromyzidae), commonly known as the potato leaf miner, is a devastating pest of *Solanum tuberosum* L. (Solanales: Solanaceae) in South Africa. The presence of *L. huidobrensis*, which originates from Central and South America, was only detected on other continents in the 1980s. After having been detected in Europe in 1987 (Lanzoni *et al.* 2009; CABI 2018), it was found to have invaded South Africa by early 2000 (Visser 2009).

Insect invasions from one country to another are inevitable. Several insect invasions have formerly been reported, mostly due to increased movement of goods and people (Pimentel *et al.* 2001; Seebens *et al.* 2018). Although many reasons contribute to insect invasions worldwide, one of the common contributing factors, influencing biodiversity distributions and invasions globally, is climate change (Hill *et al.* 2016). Most of the economic damage and crop losses result from the insect pests to which horticultural crops are no exception (Oerke & Dehne 2004). The invasions contribute to food insecurity and, thus, to an increase in poverty levels (Umesha *et al*. 2018). To reduce the crop losses associated with insect pests, synthetic pesticides have been used, which are not environmentally conscious, and which usually result in an increased number of negative impacts, like insecticide resistance, soil contamination and adverse health problems (Pretty & Pervez 2015). Integrated pest management (IPM), which is an environmentally sensitive approach, includes multiple control strategies that are effective, ecologically compatible and, above all, economically feasible, and which are geared towards meeting the needs of the producers (Norris *et al*. 2003).

Although potatoes, are native to the tropical and subtropical regions of America, they have been consumed in the Andes Mountains of the South American regions for many years (Hawkes 1990, 1994; McNeill 1999). The Europeans who travelled such areas found out about the nutritional value of potatoes, and imported them to Europe, by way of Spain's Canary Islands, as early as 1565 (Horton & Anderson 1992; Ruiz de Galarreta *et al.* 2006). The first cultivation of potatoes on the mainland in Spain occurred in 1573, where after the vegetable was disseminated to other European countries, like France and the Netherlands (McNeill 1999). From there, sailors carried potatoes with them on their voyages to India (McNeill 1999). By the seventeenth century, potatoes had already reached most parts of the world, including India, China and Japan (Hawkes 1990; Horton & Anderson 1992). Nowadays, potatoes are among the four most consumed crops worldwide (Zhang *et al.* 2017).

Potatoes are the world's number one non-grain commodity (Lutaladio & Castaldi 2009). In South Africa, they are one of the most important vegetable crops. The potato production industry contributes to 60 % of the total number of vegetables grown in South Africa and represents 3 % of the total number of agricultural products (DAFF 2012). Over 50 % of the potatoes that are produced in South Africa are consumed locally, with approximately 30 % of potatoes being exported to such nearby countries as Zimbabwe, Zambia and Mozambique (DAFF 2012). South Africa ranks 31st on the list of global production of potatoes and contributes up to 0.5 % of the total world potato production (NAMC & Commark Trust 2006). As the world population is rapidly growing, ensuring food security for the present and future remains a challenge. Potato production provides livelihoods and contributes to employment opportunities, thus helping to reduce the extent of hunger and poverty (NAMC & Commark Trust 2006).

Gaining an in-depth understanding of the biology and control options of a devastating pest insect like the potato leaf miner, *L. huidobrensis*, is an important step in implementing IPM. This review provides an overview of the current information that is available on the biology, ecology and management practices of *L. huidobrensis* under South African conditions and their potential implementation in an IPM system.

Insects affecting potatoes

Globally, 49 insect species have been recorded as targeting potato crops (Kroschel *et al.* 2020). Farmers in the tropical and subtropical regions tend to have more of a challenge in comparison to those from temperate regions, where the pest densities are generally less (Kroschel & Schaub 2013). Two key insect pests of potatoes in a number of countries are *L. huidobrensis* and *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) (Table 1.1), which cause about 70% crop loss (Picker *et al*. 2002; Visser 2005). Up to 100% crop loss has been reported in some cases (Rondon 2010; Mujica & Kroschel 2013).

Table 1. 1. Major and common insect pests of potatoes and their host range.

Scientific name	Common name	Host range
Phthorimaea operculella	Potato tuber moth	Vegetable crops mainly in the family Solanaceae
Liriomyza huidobrensis	Potato leaf miner	Highly polyphagous, recorded from 14 families
L. trifolii	American leaf miner	Highly polyphagous
Tuta absoluta	Tomato leaf miner	Attacks Solanaceae plants, including weeds
Myzus persicae	Aphids	Extremely polyphagous

Agrotis spp.	Cut worms	All vegetable crops and some cereals
Helicoverpa armigera	African bollworm	Economic host crops, including potato, pepper and celery

Leaf miners

Over the years, more than 10 000 leaf miner species have been reported worldwide from the insect orders Coleoptera, Diptera, Hymenoptera and Lepidoptera (Hering 1951). A leaf miner is an insect species whose feeding pattern occurs within the leaf tissues (Hering 1951). All the larval stages can occur inside the leaf (Visser & Schoeman 2004). During feeding, the larvae form tunnels, or mines, which are feeding channels situated inside the parenchyma, and between the epidermal tissues, of the leaves (Fig. 1.1) (Hering 1951; Basij *et al*. 2011). Both the epidermis and the outer wall remain undamaged during larval development (Hering 1951; Weintraub *et al.* 2017).

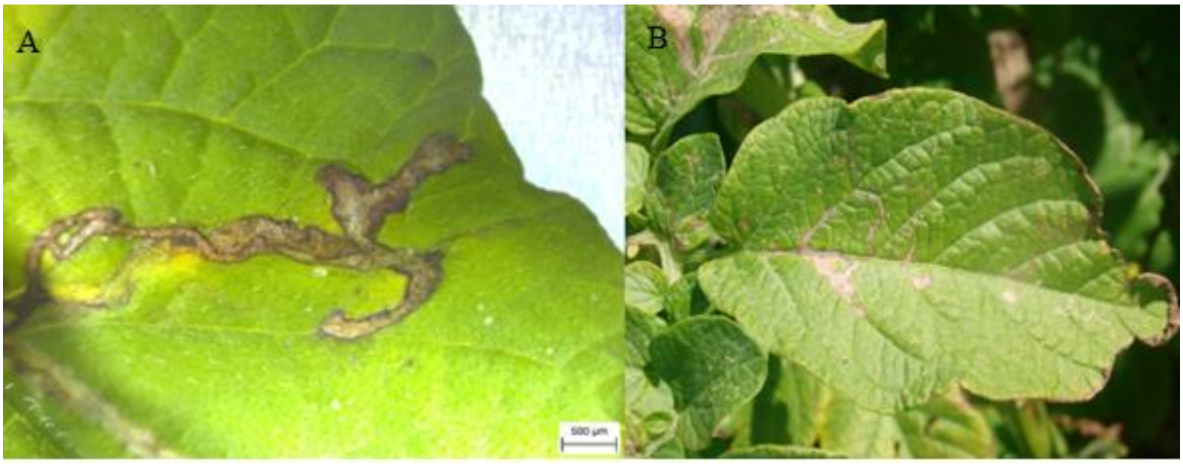

Fig. 1. 1. Leaf-mining behaviour of *Liriomyza huidobrensis* observed on an infested leaf under a microscope (A) and in a potato field (B).

The feeding pattern of the larvae is divided into two categories, facultative and obligated (Powell 1980). The facultative species consist of those that feed within the leaf and externally, in the case of the last larval instars, before they pupate, whereas the obligated species tend to feed entirely within the leaves, and may even pupate within the leaves (Hering 1951; Powell 1980; Ameixa *et al.* 2007). Other classifications of leaf miners depend on the shape of the mines, with, for example, the serpentine leaf miners obtaining their name from the zigzag pattern that is formed while they feed on the leaves (Weintraub *et al.* 2017). Some studies hypothesise that leaf-mining protects the insect pest and, more broadly, acts as a defence against several natural enemies (Hering 1951; Connor & Taverner 1997). An alternative hypothesis suggests that leaf miners might have increased susceptibility to pathogens, due to the humidity within the leaf environment, which may be conducive to pathogens (Cornell 1989). However, Connor & Taverner (1997) found that leaf-mining insects are likely to encounter a lower pathogen incidence than do those that just feed on the foliage. The success rates attained by the use of biological control options like parasitoids in relation to leaf-mining insects is greater than that which is attained with externally feeding insects (Connor & Taverner 1997).

The leaf miners' host choice is vital to the adult fly, due to the limited mobility of the immature stages, as they must feed on the plants from which they emerge (Zehnder & Trumble 1984). Thus, the quality of the host plants plays a vital role in larval development (CABI 2018). As abiotic changes may affect larval development, the adults are selective and pay close attention when choosing their hosts, with the female oviposition choice determining the larvae-feeding site and the offspring's well-being (Musundire *et al*. 2010). After fertilisation, the female leaf miner lays eggs on the plant tissue, causing oviposition punctures. Oviposition differs, depending on the insect order (Weintraub *et al.* 2017). Some orders leave puncture marks on the leaf surface after laying their eggs, while others do not do so (Hering 1951).

Potato leaf miner, Liriomyza huidobrensis

Liriomyza, which is one of the largest genera of the order Diptera (Agromyzidae), consists of over 300 leaf miner species worldwide, of which, however, only 23 are economically important (Liu *et al.* 2009). Leaf-mining occurs in nine different families of Diptera, with Agromyzidae having the largest number of species that are leaf miners (Mujica & Kroschel 2011). The leaf miners, which have invaded many different agricultural areas of the world, cause significant damage to many crops (Rauf *et al.* 2000).

Liriomyza huidobrensis is a highly invasive species, which is highly polyphagous and resistant to a variety of insecticides (Spencer 1973; Reitz *et al*. 2013; Weintraub *et al.* 2017). The potato leaf miner causes damage to crops both directly and indirectly. Direct damage is caused by the feeding and oviposition punctures of the adults (Fig. 1.2) relative to the leaf tissue. In addition, with the larvae mining from the newly hatched larvae, the mined leaves become necrotic and eventually die, due to a 62 % reduction in the photosynthetic ability of the mined leaf, which greatly reduces yield (Chabi-Olaye *et al.* 2008). Indirect damage is a result of the pathogens entering the host through perforations, which may act as pathways or vectors of plant diseases like *Alternaria alternata* (Fr.) Keissl (Pleosporales: Pleosporaceae) (Deadman *et al.* 2000). Despite *L. huidobrensis* itself not spreading pathogens, they do increase the probability of secondary plant pathogen manifestation (CABI 2018). Few studies have tried to determine the economic injury level and the economic threshold involved, but the quick life cycle and *Liriomyza* fertility makes the process challenging (Rondon 2010). Alves *et al*. (2017) obtained an economic injury level of 0.07, and an economic threshold of 0.05 mines per plant, which is very low, thus indicating the urgent need to control the insect pest.

Fig. 1. 2. Feeding and oviposition punctures of *Liriomyza huidobrensis* on the leaves of a potato plant collected from the Sandveld region, in the Western Cape province.

Pest description and biology

Liriomyza huidobrensis can be differentiated from other *Liriomyza* species by its relatively dark orange-yellow head and legs, which, in other species, are just yellow. The dark, and almost black, antennal segments can also be regarded as a distinguishing factor (Visser 2009; CABI 2018). The potato leaf miner is generally small, measuring about 2.3 mm in length, with it having a division on the second abdominal segment (Weintraub & Horowitz 1995) (Fig. 1.3A). Adult *L. huidobrensis* females can lay up to 400 eggs, measuring about 0.15 mm to 0.30 mm, with an off-white and slightly translucent colour, which hatch within a period of 2 to 5 days after oviposition (Visser 2015; CABI 2018) (Fig. 1.3B). The larvae hatch within the leaf, where they form mines while feeding (CABI 2018). The larvae undergo three larval instars: the first larval instar, which is colourless on hatching, later turns pale yellow-orange, with the late instar being yellow-orange (Weintraub & Horowitz 1995). The larvae can reach about 3.25 mm in length before pupating. The larvae of some species have been reported as leaving one leaf for another, although such behaviour has not been reported in the case of *L. huidobrensis* (Parrella

& Keil 1984; CABI 2018). After the larvae develop, the final instar larvae, after making a slit on the leaf surface to leave the mine, tend to pupate on the lower surface of the leaf, from which they fall to the soil, to complete their pupation period. The puparium, which has an oval shape (Fig. 1.3C), measures between 0.5 mm and 1.3 mm, having a brown to almost black colour (CABI 2018). The pupa pupates for a period of 10 days (Visser 2009), after which a small greyish-black fly emerges, with a body size of between 1.3 and 2.3 mm, and a wingspan of between 1.3 mm and 2.3 mm. The females are generally slightly larger than the males (Weintraub *et al.* 2017).

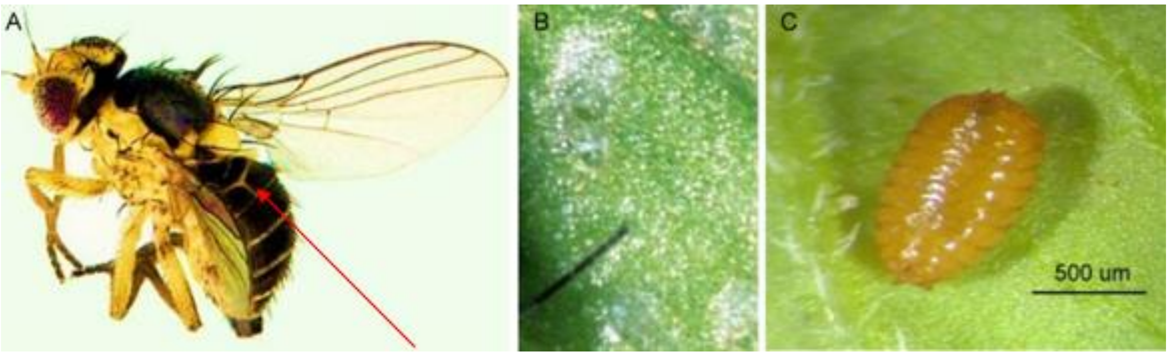

Fig. 1. 3. A, Adult *Liriomyza huidobrensis*, with a division on the second abdominal segment. B, eggs within punctures on the upper leaf. C, newly hatched pupae after leaving the leaf.

Although little information exists regarding the biology of the potato leaf miner, its life cycle is typical of all agromyzid species (Weintraub & Horowitz 1995, 1996). *Liriomyza* males are the first to emerge, followed by the females, with the mating period usually occurring 24 h after emergence. A single mating can fertilise all the eggs (Mujica & Cisneros 1997; Migiro *et al*. 2011). Most adult activity occurs in the early morning, just after sunrise, and before sunset (Weintraub & Horowitz 1995).

Host plants

Host selection is vital for different herbivorous insects, because it determines the progeny’s feeding and the female’s oviposition area (Maharjan & Jung 2016). *Liriomyza huidobrensis* is a highly polyphagous pest that can cause severe damage to many different crops (Reitz *et al.* 2013; CABI 2018). So far, 365 host plant species, from 49 different families, have been recorded (Weintraub *et al.* 2017). Only 32 % of the plant species are cultivated food crops, with most of the host plants being weeds and cultivated flowers (Weintraub *et al.* 2017). The leaf miner affects both field- and greenhouse-produced vegetable crops, ranging from being sporadic to being prevalent throughout the growing season (Reitz *et al*. 2013). The tendency of local leaf miners to have a strong preference for local plant species was observed in Argentina, where *L. huidobrensis* was found to prefer vegetable crops, like bean, beet, potato, sweet pepper and celery plants, as compared to other vegetables (López *et al.* 2010). Other studies suggest that external factors play a vital role in the host preference of *Liriomyza* flies (Fenoglio & Salvo 2009). Another study, which was conducted in China regarding host preference, found that the selectivity of the leaf miner is related to nutritional and physical factors (Liu *et al*. 2009). Other studies suggest that host selectivity does not depend on the amount of chlorophyll, soluble sugars, proteins and tannic acid concentrations present, but find, instead, adaptability to be the cause (Liu *et al*. 2009; Weintraub *et al*. 2017).

Economic importance

Female *L. huidobrensis*, during oviposition, leave feeding and oviposition punctures on the plant/leaf, which usually appear as white, round speckles (Wei *et al*. 2000; CABI 2018). The feeding puncture sizes range between 0.13 mm and 0.15 mm long, while the oviposition punctures are usually 0.05 mm long (IPPC 2016; CABI 2018). The larvae stay within their mines during their early stages. However, in pea plants, the larva also tends to feed on the outer surface of the pods (CABI 2018; Ge *et al.* 2019). A mine is usually white and turns brown as the plant grows, has an irregular shape (which is typically serpentine), and increases in size as the larva grows (Wei *et al.* 2000; CABI 2018). More than one larva may feed on any one leaf, thus leading to the production of a secondary 'blotch' mine type, and leading to the wilting of the leaf (Spencer 1973). In potato plants, the feeding punctures that are on the leaves can be used to predict an outbreak (CABI 2018). The initial larval infestation begins in the lower (third) portion of the leaves on the plant, moving to the top leaves of the plant (Visser 2005). The larval damage is most severe when the plant is full-grown, and it is less severe during the vegetative stages of the plant (Visser 2005; CABI 2018), with it having mostly been known to attack the older leaves (Visser 2009; Mujica 2016).

Liriomyza huidobrensis is included on the A2 list of quarantine pests (EPPO 2005). In South Africa today, it is one of the most important potato pests (Weintraub *et al.* 2017). Even though it is a native pest of the tropical and subtropical regions of America, its first detection on the African continent occurred as early as 1987 (CABI 2018). Some potato varieties have developed to induce ovipositor resistance against leaf miners (Videla & Valladares 2007). The resistant plants increase the multiplication rates of the leaf cells, thereby exposing *L. huidobrensis* to other predatory animals, and increasing the risk of desiccation (Videla & Valladares 2007).

Management of the potato leaf miner

Monitoring

A fundamental step in securing proper IPM is to adopt the method of monitoring. Monitoring can give the farmer the necessary information on the basis of which to make decisions pertaining to which management practices to follow (Dara 2019; Sharma *et al.* 2020). Effective monitoring practices help predict the presence of the pest, the pest numbers, and the weather conditions that favour its presence (Dreistadt *et al*. 1998; Lu *et al.* 2012). The monitoring of insect pests can be achieved by using a variety of tools, including coloured sticky traps, light traps, pheromone traps, pitfall traps, and suction traps (Epsky *et al.* 2008; McCravy 2018). No records are available regarding the use of sex pheromones for the potato leaf miner. The use of sex pheromones as a mass trapping technique to monitor the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) has, however, demonstrated great success (Braham 2014).

In the case of the potato leaf miner, the use of sticky traps coated with sticky adhesives in the monitoring of adult populations has proven to be successful; such traps have been designed in different colours, although yellow is mostly preferred (López *et al*. 2010). The use of appropriate trapping methods ultimately helps by reducing the adult pest populations and by reducing the unintended capture of natural enemies and beneficial insects, although some sticky traps end up trapping the natural enemies, as well (Chavez & Raman 1987; Lu *et al.* 2012). The plant canopy is directly related to the placement of the trap. Thus, for fast-growing plants, placement is usually a few inches above the canopy, while slower growing plants should be just above the canopy level (Dreistadt *et al.* 1998; Atakan & Canhilal 2004).

The mass trapping technique using traps could be considered a useful mode of control, according to the research done on *T. absoluta* that found no significant difference between mass trapping techniques and chemical insecticides in the control of *T. absoluta* (Braham 2014). However, only a few studies have evaluated their effectiveness (Cocco *et al.* 2012). Proper and effective monitoring enables the taking of crucial decisions and the making of recommendations on which control strategies are to be followed (Dreistadt *et al.* 1998).

Chemical control

The most common method of control used against potato pests is that of synthetic pesticides (Rondon 2010; Mujica & Kroschel 2013). However, the use of most of these insecticides can lead to the development of insecticide resistance, increased cost of production, contamination of the environment, and the killing of non-targeted organisms (Okoth *et al*. 2014). Most *Liriomyza* species rapidly develop resistance to certain conventional insecticides used in different countries. However, not all populations of the leaf miner have the same resistance profile (Weintraub *et al*. 2017). The larvae and adults are not susceptible to different insecticides, because the larval life stage is covered and protected inside the leaf, thus contact insecticides are not recommended for the control of larval populations (MacDonald 1991; Van der Staay 1992). In the early 1990s, the only effective insecticides used to control larval populations were abamectin and cyromazine (Van der Staay 1992). Other studies suggest the use of the Neem tree extract, *Azadirachta indica* A. Juss. (Meliaceae), which has proved to be highly effective against the larvae (Weintraub & Mujica 2006). Abamectin treatments are relatively expensive when compared to other pesticides, but a well-timed spray programme should enable effective and sufficient control of the potato leaf miner (Hidrayani *et al.* 2005). The Abamectin easily and rapidly breaks down when it is exposed to sunlight, and it can reach the larvae as it is a systemic insecticide and remains in the mesophyll layer of the leaf (Damavandian & Moosavi 2014). Pesticides that include pyrethroids and organophosphates are not effective against the potato leaf miner in the long run, because of resistance development (Macdonald 1991; Weintraub & Horowitz 1995).

Cultural control

Habitat management plays an important role in increasing the action of the leaf miner's natural enemies (Gurr *et al.* 2017), with some weeds acting as reservoirs for leaf miner pests (Schuster *et al.* 1991; Chen *et al*. 2003). The cultural control of *L. huidobrensis* mainly involves the use of preventive measures (Weintraub *et al*. 2017), including the constant weeding of the fields concerned (clean fields), as well as the adoption of phytosanitary measures (CABI 2018). Environment-friendly strategies (*e.g.* crop rotation, the selective removal and destruction of infested plant materials both before and after harvest, and the destruction of pupae before planting), all play an important role in suppressing leaf miner populations (Husin 2017). In terms of other crops, pruning and fertilisation have played a significant role in reducing the size of leaf miner populations (Ateyyat & Mustafa 2001; Johnson *et al.* 2011). The above supports the theory that weeds in potatoes support larger leaf miner populations (Ateyyat & Mustafa 2001). Practices like proper fertilisation could also, therefore, be used in managing potato leaf miner, since the quality of the potatoes plays a vital role in leaf miner abundance (Fenoglio & Salvo 2009).

Biological control

Many biocontrol agents have been developed in recent years (Gangwar 2017). The use of natural enemies like parasitoids, predators, pathogens, weed feeders and many more, are key elements of biological control, especially in an era where biological control options (entomopathogenic fungi (EPF), entomopathogenic nematodes (EPNs), parasitoids, viruses and bacteria) are slowly becoming more popular, and the success of the control options, consequently, reduces the use of harmful pesticides (Bhattacharya *et al.* 2003; Hassan *et al.* 2016).

Liriomyza species have a considerable cohort of natural enemies, with more than 80 different species having been reported (Liu *et al*. 2009). However, in the use of natural enemies, despite it being one of the eco-friendliest methods of control, care must be taken with introducing parasitoids into a new region through classical biocontrol programmes, because some may become pests themselves, when they are introduced to a different region from which their own natural enemies are absent (Murúa *et al*. 2006). Nevertheless, most studies suggest that natural enemies are important in regulating the *Liriomyza* species (Ode & Heinz 2002). The parasitoid, *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae), for example, is used to control agromyzid leaf miner populations in both their native and invaded areas (Rauf *et al.* 2000; Chen *et al.* 2003). In some parts of Africa, the best control of *Liriomyza* species achieved so far has been attained by using *D. isaea* through augmentative releases (Ode & Heinz 2002). Recently in Kenya, mass production systems have been developed to achieve biological control within Africa (Ode & Heinz 2002). In Germany, the most used parasitoid against *Liriomyza*, in greenhouses, is *Dacnusa sibirica* Telenga (Hymenoptera: Braconidae) (Leuprecht 1992). However, its effectiveness depends on the number of releases achieved per week (with between three and four releases being recommended) (Leuprecht 1992). Other studies conducted in German-based greenhouses suggest the use of *D. sibirica* in combination with *Opius pallipes* Wesmael (Braconidae**:** Hymenoptera) for effective control (Van der Linden 1991).

The use of biological control options helps in providing a stable and environment-friendly pest management programme. The implementation of such options, in the form of management programmes, can potentially reduce the need for chemical insecticides, and the subsequent residual side-effects thereof, in agriculture (Hajek & Delalibera 2009). The development of fungi as biocontrol agents against different pests, weeds and diseases has been an area of interest in recent years (Butt *et al*. 2001). Several EPF are common, and due to them being known to induce epizootics, they are, thus, very important in terms of regulating insect populations (Butt *et al.* 2001). EPF invade their hosts through the external cuticle, with some species being able to infect their hosts through their digestive tracts (Bonnie *et al*. 2004; Zimmermann 2007). The infestation process usually starts when the spores attach themselves to an insect's cuticle (Altinok *et al.* 2019). They then germinate and penetrate the integument, through enzymatic degradation of the cuticle and physical pressure (Butt *et al*. 2001; Hajek & Delalibera 2009). After spore penetration, the fungi produce mycelia, which then ramify within the host haemocoel (Altinok *et al.* 2019). Due to the depletion of nutrients and the action of fungal toxins, the host then dies (Butt *et al*. 2001; Bonnie *et al.* 2004). Under certain conditions, hyphae, emerging from the dead cadavers, may produce spores (Butt *et al*. 2001; Goettel *et al.* 2008).

The insect parasitic nematodes of Steinernematidae and Heterorhabditidae have been actively used since the 1990s (Poinar 1990; Navon & Ascher 2000). EPNs have been reported to show potential for use in different management strategies (Platt *et al.* 2020), due to their ability to locate, infect and kill several insect species actively (Campbell & Lewis 2002). The nematodes are obligate pathogens in nature, possessing a non-feeding phase that is also known as the infective juvenile (IJ) stage (survival third-stage larvae), a free-living phase, which is the only stage that can infect the insect host in soil substrates, as well as being able to survive outside the host (Stock *et al*. 1999; Hazir *et al.* 2004). The IJs are only able to infect a host through such natural openings as the mouth, the anus and the spiracles (Campbell & Lewis 2002; Hazir *et al*. 2004). After penetration, the IJ releases a mutualistic bacterium, either through the anus or the mouth, depending on the genus (Kaya & Gaugler 1993; Hazir *et al*. 2004). The released symbiotic bacteria colonise the insect and kill it within 1 to 2 days. The nematode then feeds on the bacteria and the bioconverted tissue of the dead larvae, undergoing two to three generations over a period of 1 to 2 weeks within the dead insect's body, depending on its size (Gözel & Gözel 2016).

EPNs to control leaf miners

Following the degree of success attained in the use of EPNs against soil-based insect pests, their use as pest control agents of foliage pests has dramatically increased (Lacey & Georgis 2012; Platt *et al.* 2020). Several advantages make it easy for EPNs to be widely used for controlling a variety of insect pests (Kerry & Hominick 2002), including their narrow host range, their non-contamination of the environment and their ready production on a large scale (Kerry & Hominick 2002; Dunn *et al.* 2020). Due to such advantages, several studies have been conducted that have demonstrated the potential of EPNs as biocontrol agents, and that have enabled the study of the susceptibility of insects to different EPNs during various larval stages. EPNs can enter the mines infested by leaf miners through oviposition sites and feeding punctures formed by adults, making foliar application viable (Harris *et al.* 1990; Steyn *et al.* 2019).

Tuta absoluta is an important pest of tomatoes, with synthetic pesticides having been used worldwide for their suppression (Siqueira *et al.* 2001; Batalla-Carrera *et al.* 2010). Many cases of insecticide resistance have, however, been documented (Siqueira *et al*. 2001; Silva *et al*. 2016). Recent studies of EPNs against *T. absoluta* larvae, both in the soil and on leaves, show that most of the nematodes have shown potential for use as biocontrol agents, with high mortality having been recorded (Batalla-Carrera *et al.* 2010). The use of EPNs and EPF in management programmes has demonstrated high mortality potential at low concentrations, both under laboratory and glasshouse conditions. Kamali *et al*. (2018) tested two EPN species, *Steinernema carpocapsae* (Weiser 1955) Wouts, Mráček, Gerdin & Bedding (Nematoda: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar (Nematoda: Heterorhabditidae), against last-instar *T. absoluta* larvae at three different temperatures (19 °C, 25 °C, and 31°C). The results indicated excellent performance for all EPNs at all temperatures, recording high mortalities of between 76 % and 96 %. Nematode performance is determined by several factors, including temperature. In a study conducted by Lortkipanidze *et al*. (2019), the effect of temperature on the virulence of EPNs was determined. The study recorded the responses of selected nematode species to various temperatures, with some species being found to perform very well at relatively high temperatures. Such findings provide evidence that temperature is an important environmental variable, affecting the overall fitness of nematodes (Lortkipanidze *et al.* 2019).

Williams & Walters (2000) conducted a study to determine the susceptibility of three leaf miner species to *Steinernema feltiae* (Filipjev 1934) Wouts, Mráček, Gerdin & Bedding. All three species, consisting of *L. huidobrensis*, *Liriomyza bryoniae* Kaltenbach (Diptera: Agromyzidae, and *Chromatomyia syngenesiae* Hardy (Diptera: Agromyzidae), were highly susceptible to the EPN. A reduction was shown in pupal production from the larval stage onwards, in comparison to the performance of the control treatment. In a South African study, Steyn *et al.* (2019) investigated the potential of South African EPNs to control the leaf miner, *Holocacista capensis* Van Nieukerken & Geertsema (Lepidoptera: Heliozelidae). The study was aimed at determining the susceptibility of the leaf miner to seven locally isolated EPN species. The bioassays showed that the local isolates of *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens, *Heterorhabditis indica* Poinar, Karunakar & David and *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt were the most virulent species, as well as demonstrating the ability to penetrate mines and to infect the leaf mining.

Nematode survival is affected by different abiotic factors (*e.g.* temperature and humidity) which explain why controlling the above-ground insect life stages under field conditions is difficult (Grewal *et al.* 1994; De Waal *et al.* 2013). Accordingly, a study conducted by Platt *et al*. (2019a, b) was directed at investigating the foliar application of *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams to control *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) and at assessing the use of adjuvants to improve efficacy (Platt *et al.* 2018). In the study, two commercial adjuvants, Nu-Film-P® and Zeba®, were added to the nematode suspension. The results indicated that the inclusion of adjuvants in nematode foliar application resulted in an increased number of IJs being deposited on grapevine leaves, with significant differences being found between the different treatments. The use of both adjuvants combined was the most effective in terms of retaining nematodes on grapevine leaves, followed by Zeba® alone, and then by Nu-Film-P®, with the control treatments being found to be the least effective (Platt *et al*. 2019a, b).

IPM is such a comprehensive concept that it includes many different components, such as the use of synthetic insecticides in combination with different biocontrol agents. Various studies have been conducted to determine the compatibility of *S. feltiae* and chemical insecticides in terms of the control of *L. huidobrensis*. Head *et al.* (2000) used five insecticides (abamectin, deltamethrin, dimethoate, heptenophos and trichlorfon), with the results obtained showing that abamectin, deltamethrin and heptenophos reduced the ability of the nematode to locate and infect hosts. However, as no negative effects on the nematodes were recorded when trichlorfon and dimethoate were used, they are regarded as a superior choice when considering combining biocontrol agents with synthetic insecticides in a potential management programme (Head *et al.* 2000).

EPF to control leaf miners

EPF are another alternative regarding the control of sap-feeding insects (Inbar & Gerling 2008). A major objective of biocontrol agents is to reduce the number of crop pests present without contaminating the environment and disturbing other organisms (Ooi 2000). At present, as environmental contamination is reaching greater heights than before, an increasing number of studies are being conducted to investigate the possibility of using biocontrol agents against leaf miners (Abd El-Salam *et al.* 2013). Various strains of *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) have been reported as being virulent against dipteran pests. However, few attempts have been made, as yet, to investigate the use of EPF against dipteran leaf miners (Quesada-Moraga *et al*. 2006).

Consistent results have been reported on the pathogenicity and virulence of *M. anisopliae* to *T. absoluta* third-instar larvae, indicating its potential for use as a biocontrol method for the control of other leaf miners (Inanl & Oldargc 2012; Alikhani *et al.* 2019). Both *Metarhizium* and *Beauveria* species have been documented as being pathogenic towards eggs and larvae (Inanl & Oldargc 2012). However, not all leaf miners can be controlled by EPF and EPNs (Progar *et al.* 2015). Witness, for instance, the reported increase in the number of invasive leaf miner, *Profenusa thomsoni* (Hymenoptera: Tenthredinidae), after the application of different fungi isolates, which was reported as occurring in a study, conducted in Alaska, to determine the feasibility of applying *B. bassiana* and *S. carpocapsae* to the soils at different sites maintained under field conditions (Progar *et al.* 2015). Despite the success of entomopathogens in most other studies, the results obtained in the above study poorly supported the idea of their pathogenicity. Instead, strong evidence came to light regarding an increase in pest density at the evaluated sites, although it was unclear which factors contributed to this (Progar *et al.* 2015). The EPNs that have been reported to infest *Liriomyza* species include *B. bassiana*, *Isaria* (= *Paecilomyces) fumosoroseus* Wise (Hypocreales, Clavicipitaceae), *Paecilomyces lilacinus* (Thom) Luangsaard, Houbraken, Hywel-Jones & Samson (Hypocreales; *Cordycipitaceae*, *M. anisopliae* and *Verticillium lecanii* Zare & Gams (Hypocreales: Cordycipitaceae (Liu *et al.* 2009). *Metarhizium* and *Beauveria* species were found to be the most used EPF in previous studies (Gürlek *et al.* 2018).

A study by Migiro *et al.* (2011), investigating the pathogenicity of *M. anisopliae* and *B. bassiana* isolates to adult *L. huidobrensis* under laboratory conditions, found that all 17 isolates evaluated were pathogenic to the leaf miner, causing mortalities of between 40 % and 100 % after five days' exposure. The minimum lethal time ranged between 2.6 and 5.4 days, causing 50 % mortality, depending on the isolate.

In a study conducted by Noujeim *et al*. (2015), pathogenicity tests using *B. bassiana* were used to determine the effect of EPF and EPN (*Heterorhabditis indica*) on *L. huidobrensis* pupae. The results showed mortalities ranging between 73 % and 97 % at different concentrations. The finding paves the way for additional research regarding the susceptibility of the *L. huidobrensis* pupae. Migiro *et al.* (2011) suggest a reduction in the oviposition potential of *Liriomyza* flies due to fungal infection, thus supporting the use of entomopathogens for the control of *Liriomyza*, since such control is possible at all life stages.

Parasitoids to control leaf miners

The many cases of success in the use of parasitoids to control different leaf miner pests can be attributed to the abundance of parasitoids associated with leaf miners (Rauf *et al.* 2000; Chen *et al.* 2003). In a survey on the species composition of the host crops of leaf miners and parasitoids in Indonesia, the most common parasitoid species associated with *L. huidobrensis* was found to be *Hemitarsenus varicornis* (Hymenoptera: Eulophidae), with a 92 % infestation record (Rauf *et al.* 2000). One of the most often used parasitoids around the world is *D. isaea*, which is a solitary larval ectoparasitoid of a variety of leaf miner species (Ode & Heinz 2002; Liu *et al*. 2009).

Studies on the rates of parasitism of leaf miners in different environments, including those near forests and in villages and forests, as well as in urban parks and streets, were conducted for both first- and second-generation parasitoids, with the conclusion being drawn that no significant difference was found between the two generations. However, the rate of parasitism increased during the pupation stage in the first generation and decreased in the second generation. Furthermore, no significant difference was found between the mines of the host plants that were evaluated in the different environments (Girardoz *et al*. 2006).

Parasitoids are affected by several different factors, both abiotic and biotic, with thermal conditions being found to be key (Rousse *et al.* 2009). Sugimoto *et al.* (2006) compared the thermal tolerance of different native species of parasitoids in Japan when acting as biological control agents against the leaf miner *Liriomyza trifolii* (Burgess) (Diptera: Agromyzidae). A decrease in the length of the development period was observed, as the temperature rose above 25 °C. At temperatures of above 30 °C, only male, but no female, parasitoids emerged. The study also investigated the effects of temperature on host feeding and parasitisation, which differed, depending on the evaluated temperatures and the exposed parasitoid species concerned (Sugimoto *et al*. 2006).

Alternative control strategies

Sterile insect technique (SIT) is an environmentally conscious control strategy that aims to reduce pest populations through releasing overwhelming numbers of sterile male insects (Dyck *et al.* 2005). The population is suppressed through the sterility of the F1 generation, which, if the individuals concerned mate with a wild female, produces non-viable offspring (Knipling 1955). Few reports suggest the use of SIT as a control strategy of the potato leaf miner. However, Kaspi & Parrella (2006) obtained successful control of *L. trifolii* after an augmentative release of parasitoids and sterile males. SIT has been used in many lepidopteran insects and on the tomato leaf miner, *T. absoluta* (Tarusikirwa *et al.* 2020). Given the efficacy of SIT in the pest management of other insects, its soundness with regards to the environment, and its compatibility with different control measures, requires exploring in terms of its use in potato leaf miner management in South Africa, especially in light of it being a very challenging pest to control (Weintraub *et al.* 2017). The use of sex pheromone-based strategies, like mass trapping and mating disruption, are promising techniques for use against *T. absoluta* (Tarusikirwa *et al.* 2020). Such findings provide insight into the potential of using the two techniques against the potato leaf miner, which feeds in a similar way.

CONCLUSION

Food security has become an important issue worldwide. However, it cannot be guaranteed if crops are constantly under attack by both pests and diseases. Such an onslaught makes sustaining agricultural productivity difficult, especially where there is a dearth of research and innovation, because pests, which are constantly developing resistance to synthetic pesticides, are continuously spreading to new areas around the globe. An increase in the use of synthetic insecticides has been reported over the years, with most of the leaf miners developing resistance against several active ingredients. Such behaviour calls for an increase in the number of IPM practices, including in those supporting the use of EPF and EPNs, with such use having been proved to be sustainable in the long term, as it avoids contamination of the environment. The possibility that EPNs, EPF and parasitoids can infect and colonise all the life stages of the leaf miner is a cardinal point to consider, requiring conformational research. Although previous studies on the effects of biologicals on the potato leaf miner have concentrated on some of the larval stages, not all of the life stages have been investigated in depth. For instance, in South Africa, only a few studies have been conducted regarding the potato leaf miner, especially in the Western Cape province. Therefore, the current review combined the available information regarding the alternative methods of pest control of the potato leaf miner *L. huidobrensis* that are available to farmers, by means of evaluating its susceptibility to different biocontrol agents.

AIM OF THE STUDY

The study aimed to obtain information on the bio-ecology of *L. huidobrensis* and provide preliminary data on biological control agents in the potato fields of Western Cape province. The objectives of the study WERE:

- Survey potato fields in the Sandveld district, to confirm the leaf miner species causing damage and identify potential parasitoids; and investigate the feasibility of establishing a colony of the dominant species found;

- Determine the mode of infestation of potato fields, whether from surrounding natural vegetation, or from within potato fields (e.g. previous plantings);

- Screen potato field soil for potential soil-borne pathogens (EPNs and EPF) using bait insects (mealworm larvae);

- Evaluate the susceptibility of the potato leaf miner to EPNs and EPF, to be used as biocontrol agents.

As the thesis appears in the form of three individual journal publications, some repetition might occur between the different chapters. The format of the journal *African Entomology* was followed.

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CHAPTER 2

Occurrence and Distribution of the Potato Leaf Miner, *Liriomyza huidobrensis* (Diptera: Agromyzidae) and Parasitoids in Potato and Natural Vegetation of South Africa

ABSTRACT

The leaf miner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) is a polyphagous fly, infesting a wide range of vegetables and ornamental plants. However, there is little to no knowledge of the biology and ecology of this pest in the Western Cape province of South Africa, both of which are essential components in developing an effective management program. Several aspects of its biology and ecology were studied in two potato, *Solanum tuberosum* L. (Solanales: Solanaceae), fields in the Sandveld region during the winter and summer period. Population densities of adult *L. huidobrensis* were monitored by using yellow sticky traps, which were monitored and counted once every two weeks. The number of leaf miners caught throughout the summer and winter monitoring period were significantly different ($F_{5, 220}$ = 17.170, P < 0.001). In addition, 50 randomly selected plants were checked for leaf damage (punctures and mines) using presence/absence sampling. Leaf miner persistence within the soil was confirmed through one-time soil sampling of the two fields one month after harvesting. The leaf miner pupae were detected in the soil samples after potato harvest. However, no adult emergence was recorded among the collected pupae under laboratory conditions. The observed trend was that traps in both fields caught more leaf miners as the crops matured, damage occurred in both the monitored fields in as early as week four after plant emergence. Of the five different parasitoid species collected *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae) was the only abundant parasitoid found. Assessing the abundance, indicated *L. huidobrensis* to be present in the potato fields of the Sandveld, South Africa and that adult populations increased with crop development.

Key words: *Liriomyza huidobrensis*, parasitoids, plant emergence, *Solanum tuberosum*, traps

INTRODUCTION

The potato, *Solanum tuberosum* L. (Solanales: Solanaceae), is considered an essential food tuber around the world, grown in more than 125 countries by both subsistence and commercial farming (Caldiz *et al*. 2009). In South Africa, the annual planted area with potatoes is between 50 000 and 54 000 ha, distributed throughout the country, though mostly in Limpopo, Western Cape, Free State and Mpumalanga provinces (DAFF 2012). However, potato production is subjected to attack by several insect pests that affect productivity, which results in considerable crop damage (Oerke & Dehne 2004). The potato leaf miner, *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae), is an important pest of potatoes, causing substantial damage in different parts of the world (Reitz *et al*. 2013). It is known to affect several plant species worldwide, due to its polyphagous behaviour (CABI 2019), affecting mostly vegetable crops and weeds (López *et al.* 2010; Reitz *et al*. 2013). The term leaf miner refers to insects that have one or more of the life stages that occur inside the leaf tissue. The leaf-mining habit makes it vulnerable to predators, because when within the leaves, there is limited ability to escape predation (Visser & Schoeman 2004). However, other studies suggest that the leaf-mining habit is a mechanism used by the insect pest as defence against other insect pests (Hering 1951; Connor & Taverner 1997). An alternative notion is that leaf mining insects are likely to be susceptible to pathogens due to the humidity within the leaf (Cornell 1989).

Mining activities reduce the photosynthetic potential of the infested plant leading to plant mortality (Chabi-Olaye *et al*. 2008). Therefore, for effective control options, chemical insecticides have been widely used, usually applied after noticing the first signs of infestation (Rondon 2010; Mujica & Kroschel 2013). *Liriomyza huidobrensis* population densities increase rapidly, Weintraub & Horowitz (1995) reported increase in population densities from 38 % in week 5 to 46.7 % in week 9 after planting in a study conducted in Peru. The increased populations result in the frequent application of synthetic insecticides on the potato plants in large quantities (Reitz *et al*. 2013). These applications elevate production costs and cause negative impacts, including a natural enemy reduction and contamination of the environment (El-Wakeil *et al*. 2013). It has been recognized in some studies that outbreaks are associated with synthetic insecticide overuse (Vincini & Carmona 2006), because natural enemies are lost and thus, increase leaf miner reproduction (Johnson *et al.* 2002).

Previous studies have demonstrated that natural enemies play a vital role in regulating the population of *Liriomyza* species in their native areas, as well as invaded areas (Rauf *et al*. 2000;

Chen *et al.* 2003). To reduce the use of synthetic pesticides, biological control options (like the mass release of parasitoids) have become popular alternatives (Connor & Taverner 1997). There have been several parasitoids recorded on the potato leaf miner in different parts of the world (Civelek *et al*. 2002).

The parasitoids recorded on *Liriomyza* species mostly include families of the order Hymenoptera: Braconidae, Eulophidae, and Pteromalidae (Shepard & Braun 1998; Weintraub & Horowitz 1998; Civelek *et al*. 2002). However, one of the most used parasitoids around the world is *Diglyphus isaea* (Walker) (Hymenoptera: Eulophidae), a solitary larval ectoparasitoid of different leaf miners including *L. huidobrensis, Liriomyza sativae* Blanchard and *Liriomyza trifolii* (Burgess) (Ode & Heinz 2002; Liu *et al.* 2009). The use of parasitoids as biocontrol agents has become popular in different parts of the world (Europe, USA, Asia, and in recent years in different parts of Africa) (Ode & Heinz 2002; Liu *et al.* 2009; Musundire *et al*. 2012). Different studies show the efficacy of parasitoids in regulating leaf miner populations, and so far, there are more than 300 known parasitoid species that are associated with agromyzids. However, of these, only 80 species are associated with *Liriomyza* species (Liu *et al*. 2009; Mujica & Kroschel 2011).

The effectivity of parasitism depends on the ability of the parasitoid to locate suitable hosts and kill the larvae through the consumption of the host (Kruidhof *et al*. 2019). Therefore, the highest success with the use of *D. isaea*, for example, as biological control has been through augmentative releases (Ozawa *et al.* 2001). Some studies suggest that parasitoid efficiency varies depending on the species, differs between different life stages of the same species, and is affected by environmental conditions (Grabenweger *et al.* 2010). Environmental conditions are believed to play an important role, as higher insect mortality was reported as a result of parasitism in temperate regions, as compared to tropic regions (Hawkins *et al*. 1997; Klock *et al*. 2003; Hill *et al*. 2016). The rate of parasitism may also be affected by the presence of other organisms (Fenoglio & Salvo 2009; Fenoglio *et al*. 2012). Johnson & Hara (1987) argue that the effectivity of specific biological control agents may also depend on the plant species on which the leaf miner feeds.

Finally, there have been successful examples of classic biological control using parasitoids on leaf miners, both in the open fields and in greenhouses (Dharmadhikari *et al*. 1977; García-Marí *et al.* 2004; Cusumano *et al*. 2020). However, several aspects must be considered, such as tolerance to humidity, the ability to parasitize the host, and host synchronization (Wang *et al*. 1999; Girardoz *et al*. 2006). Changes in temperature result in the inability of the parasitoids to parasitize successfully (Kalaitzaki *et al*. 2014; Duan *et al*. 2014).

Environmental variables such as temperature, precipitation, atmospheric pressure, turbulence, and UV radiation may potentially influence species distribution and community structures of the potato leaf miner (Hodkinson 2005). However, the direct effects of these environmental factors may not explain their distribution patterns geographically (Lomolino 2001; Eyre *et al*. 2005). Recent studies indicated that factors like thermo tolerance are essential when determining the seasonal abundance and geographical distributions of most *Liriomyza* species (Rodríguez-Castañeda *et al.* 2017). However, there is no sufficient information regarding the direct effects of both abiotic and biotic factors on leaf miner abundance and survival. In this regard, local information regarding the ecology and biology of the leaf miner is not available (Jeffs & Lewis 2013). Therefore, it is important to have knowledge on the mode of infestation of the potato leaf miner, such as whether they come from surrounding vegetation, their population dynamics, overwintering mechanism and damage levels.

The study aims to gain insights into the overall presence and distribution of leaf miner and their parasitoids in potato fields in the Western Cape province, South Africa. This was accomplished by monitoring different potato fields by observing the mode of infestation on infested leaves and through trap collections. This information can help fine-tune management programmes to improve control strategies against the pest in the Sandveld region of the Western Cape province.

MATERIALS AND METHODS

Climatic conditions of the Sandveld

The Sandveld is a region found in the Western Cape, South Africa. It is a narrow area situated between the Swartland and the West Coast. The region experiences a typical Mediterranean climate with hot, dry summers and winters which are moderate to cold and wet. The average rainfall experienced within this region ranges between 300 and 400 mm per annum in winter months (between April and September) (Schulze *et al.* 2008). The mean annual temperatures range between 16 °C and 19 °C, with temperatures reaching up to 35 °C in summer and a low of 8 °C in winter (Schulze *et al.* 2008). Evaporation rates range between 5.5 and 7.35 mm/day in summer and 1.5 and 2.3 mm/day in winter, with values increasing towards the coast

(Schulze *et al*. 2008). The estimated irrigation requirements for potatoes in the Sandveld is about 700 mm for crops planted in September and about 610 mm for crops planted in August. However, the irrigation requirements will vary according to rainfall, temperature, and wind (Schulze *et al*. 2008; DAFF 2012).

Field Sites

For this study monitoring took place in the Sandveld region, which is the largest potato growing area in the Western Cape. The study sites were on three different locations which include: Saamstaan (32°35'19.3"S 18°20'07.9"E and 32°34'50.8"S 18°20'05.4"E) the two sites used to determine the mode of infestation. Modderfontein (32° 34'23.8"S 18°22'47.1"E), Taaiboskraal (32°33'18.3"S 18°24'38.3"E, 32°33'50.6"S 18° 24'25.9"E, 32° 33'47. 3"S 18°25'39.6"E and 32°33'32.7"S 18°25'59.0"E), which were used as leaf collection sites. The leaves were used for the parasitoid survey and from which colony material was collected (Fig. 2.1). All fields were commercially produced with standard fertilization, pest management and irrigation programmes. Lastly, alternative host survey was conducted in Philippi, Cape Town, South Africa (34°02'23.9"S 18°32'42.1"E).

Fig. 2. 1. Map showing study sites for the survey of the potato leafminer *Liriomyza huidobrensis* and its parasitoids in the Western Cape province.

Adult population dynamics

Two potato fields on the farm Saamstaan were regularly sampled for adult potato leaf miner and larval damage for 12 weeks. Monitoring involved placing yellow sticky traps in potato fields at specific distances (25 m, 50 m and 100 m) from the centre point following transects running from the centreline to the edge and into the surrounding natural vegetation. Each potato field comprised four such transects, and each contained six sticky traps traps in total, three within the potato fields and three in the surrounding natural vegetation. The traps were placed at the beginning of the growing season (winter: June 25th to October 1st and summer: October 1st to January 23rd) and monitored and replaced every fortnight. After the replacement of traps, the old traps were taken to the laboratory and the flies on the sticky pad were counted and morphologically identified. Identification of leaf miners was carried out using a dissection microscope with the aid of a taxonomic key (Weintraub 2001).

The traps used in this study consisted of a metal rod of approximately 4 mm in diameter. The yellow sticky traps from Chempac Pty. Ltd. (Simondium, South Africa) were attached to the metal rods using cable ties, tightened to allow the traps to rotate around the horizontal plane freely (Fig. 2.2). The design permitted vertical and horizontal rotation in the windy conditions that prevailed in the region, which prevented folding of traps. The soils of the Sandveld are sandy and soft, therefore, to reduce the risk of traps been blown away, white PVC pipes were inserted into the soil, and the metal rod placed into the top opening, allowing rotation of the trap in all wind directions. The height of the trap was moved slightly above the canopy level every two weeks to enable the flies to see the traps.

During sampling, the phenological stage of the crop, weed occurrence and the status of the surrounding vegetation, which was mainly composed of daisies *Arctotis breviscapa*, Saldanha Pincushion (*Leucospermum tomentosum*), graafwater shrubs, *Brachylaena discolor* and *Helichrysum* plant species were recorded.

Fig. 2. 2. Trapping design: A. Sticky trap attached to the metal rod with cable ties and the green lids in the sides were to prevent traps from folding and B yellow sticky traps with insects.

Damage assessments and persistence after harvest

A damage assessment was conducted in the two fields which involved random sampling of 50 plants within the field every two weeks (adapted from Chiluwal *et al.* 2012 who sampled 10 plants), checking for the presence of feeding larvae on the lower leaves, from the second week after plant emergence until harvest. If the leaves showed any sign of damage, it was noted as being present, following a presence/absence sampling method. The evaluation of leaf miner persistence in the soil after harvesting, included 10 × 1 kg soil samples collected randomly from potato fields a month after harvest from the same two fields. Soil samples were then taken to the laboratory, and they were subsequently checked for the presence of pupae using a microscope. Thereafter the pupae were incubated at 25 °C to allow eclosion of adults, this was done with the intention of confirming viability.

Insect rearing and parasitoid survey

The initial colony was started from the leaf samples collected from different fields of the Sandveld. The infected leaf samples underwent incubation for six days at 25 °C and long photoperiod (16 L: 8 D) in ventilated containers at the insectary (the insectary is located at Stellenbosch University Department of Conservation Ecology and Entomology). After 6 days, pupae formed and were stored in Petri dishes until adult emergence. Perspex cages (60 cm length × 60 width × 60 cm height) were used to house the emerging adults. Here they were able to lay their eggs on potted bean plants, tomato plants and various weed species. The potted plants were checked four times a week and replaced if the leaf surfaces were punctured all around and new potted plants were added for adult feeding. The potted plants were kept at a temperature of 25 ± 2°C, and long photoperiod (16 L: 8 D). 60 % ± 5 % relative humidity (RH) within the cages was maintained with wet paper towels and cotton wool placed inside the cages. The surrounding humidity in the insectary was between 70 % and 75 %. Temperature and photoperiod conditions were controlled using the PlantVisor process monitor, while the humidity was measured using an HTC-1 temperature and humidity thermometer indoor clock. Any emerging parasitoids were identified and stored in 80 % ethanol. Identification of parasitoids was through morphological keys (Rakhshani *et al.* 2011; Rakhshani *et al.* 2012) and DNA sequencing. All DNA sequences were performed by Inqaba biotec and the sequences were verified and edited using the CLC DNA Workbench software and to validate species identity the NCBI Taxonomy database was used. The parasitoid survey was conducted three times during the winter monitoring period.

Alternative host survey

To determine if there are any alternative hosts for the potato leaf miner, random fields containing different vegetable crops and weeds from Philippi, Cape Town, South Africa (34°02'23.9"S 18°32'42.1"E), were sampled by collecting 50 infected leaves from different crops and weeds to assess the host plant status of *L. huidobrensis* in the Western Cape. The sampling of the host plants and weeds took place twice, on 21 March 2019 and 22 October 2019. The infected leaves were incubated in the laboratory as described above, and emerging flies identified. The survey was conducted in Phillipi, because of reports of leaf-mining insects activity in different crops and vegetables in the area. Thus, damage by the potato leaf miner or other leaf miner species was investigated.

Off season sampling of potato leaf miner in the natural vegetation

The natural vegetation near the potato field on the farm located at (32°35'19.3"S 18°20'07.9"E) was sampled for adult potato leaf miner for 12 weeks after potato harvest. Monitoring involved placing 12 yellow sticky traps in natural vegetation at specific distances (25 m, 50 m and 100 m) following the same experimental design used during monitoring of adult population dynamics. Every two weeks the traps were replaced, and the old traps were taken to the laboratory to be counted and identified.

Data analyses

All analyses were conducted using STATISTICA 13.3 (TIBCO Soft Inc. 2016). To evaluate the abundance and distribution of the leaf miners, a multiple regression analysis was conducted to determine correlation between the means and one-way ANOVA (analysis of variance) & VEPAC comparison of means of different locations, weekly evaluation, and field status (at 95 % confidence intervals) was conducted.

RESULTS

Adult population dynamics

Leaf miner populations were low during the winter monitoring period, the highest catches of the leaf miner flies in the potato field were between week 10 and 12 with mean trap catches of 126 (± 11.74) and 135 (± 11.74), respectively. Whereas the natural vegetation had very low leaf miner numbers with the highest mean catches being between week 10 and 12 as well with mean catches of 7 (± 11.74) and 8 (± 11.74) flies. The mean number of flies caught differed significantly ($F_{5,220}$ = 17.170, $P < 0.001$) between the actual potato field and the natural vegetation (Fig. 2.3).

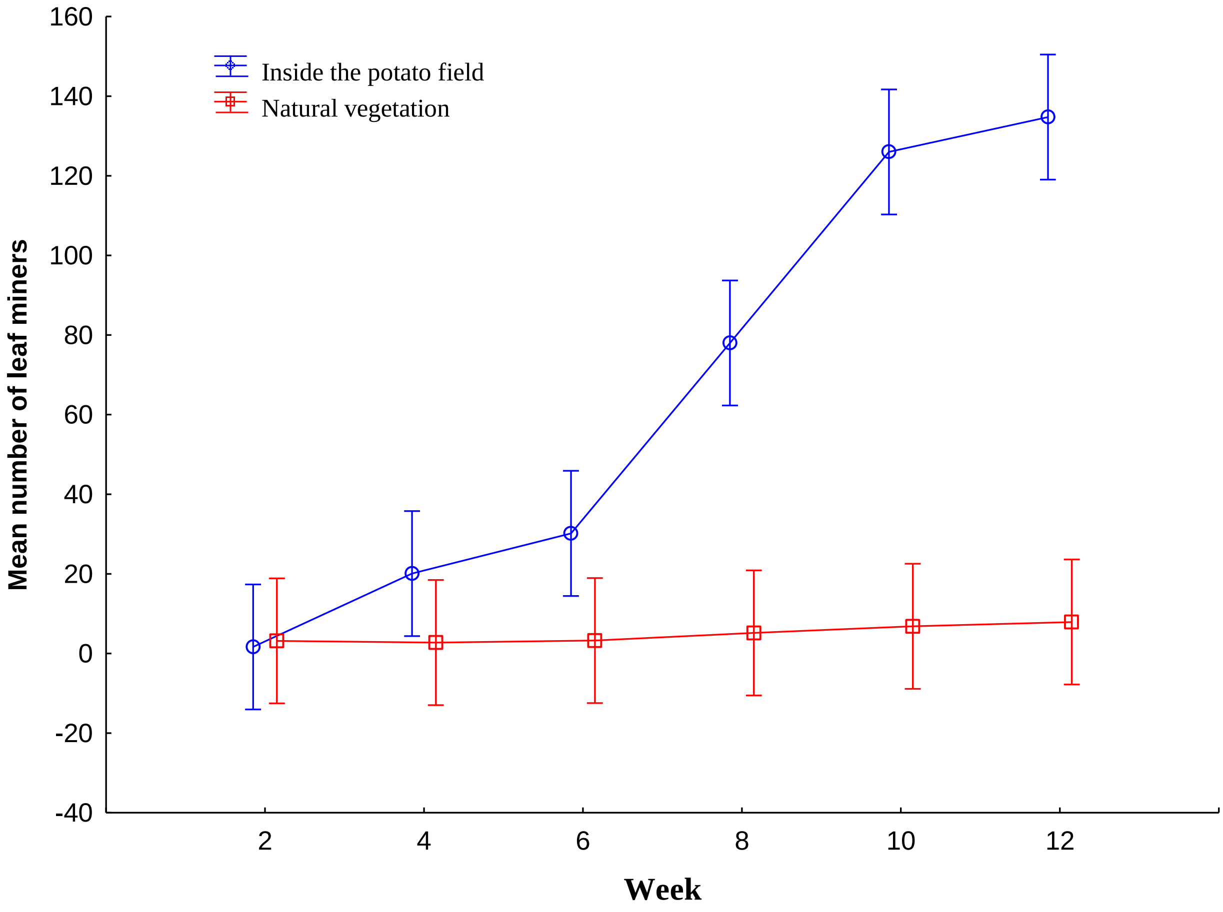

Fig. 2. 3. Mean number of adult leaf miners caught on yellow sticky traps on fortnightly basis during the winter growing season in the potato fields and adjoining natural vegetation.

During the summer monitoring the highest catches of the leaf miner flies were between week 8 and 10 with mean trap catches of 311(± 15.05) and 312 (± 15.05), respectively (Fig. 2.4). However, a subsequent reduction in leaf miner catches was observed as the plants matured with mean trap catches reducing to 167 (± 15.05). The results also indicated significant differences between the amount of leaf miners caught within the potato field and natural vegetation ($F_{6,132} = 38.462$, $P < 0.001$).

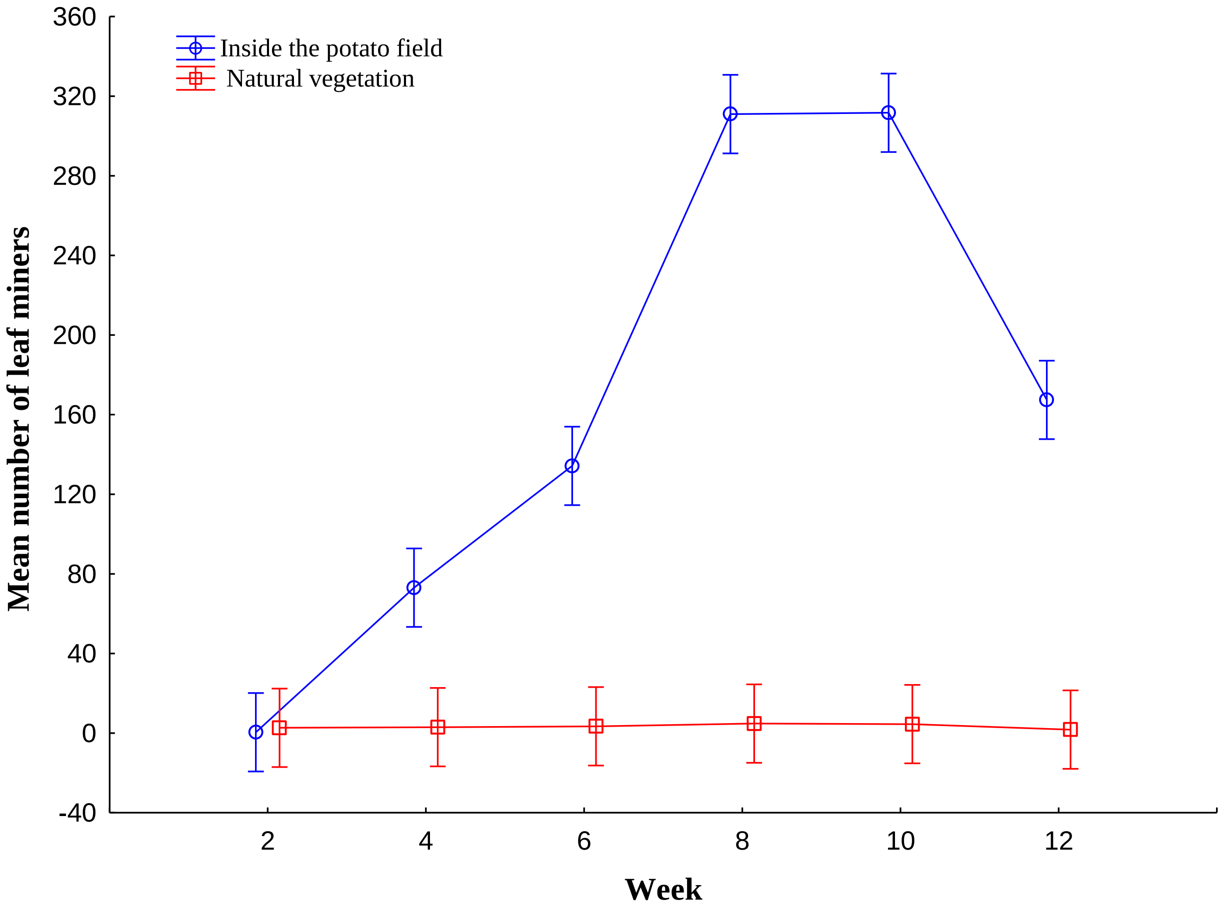

Fig. 2. 4.Mean number of adult leaf miners caught on yellow sticky traps on fortnightly basis during the summer growing season in the potato fields and adjoining natural vegetation.

The results showed that the mean weekly trap catches of leaf miners differed significantly ($F_{5, 220}$ = 17.170, P < 0.001) between the two fields (winter: 25 June to 1 October and summer: 1 October to 23 January). However, there were no significant differences (P = 0.43) observed in the population densities of leaf miners in surrounding natural vegetation at the beginning of the planting season and before harvest (Fig. 2.5). A build up in leaf miner densities was observed during both winter and summer monitoring period.

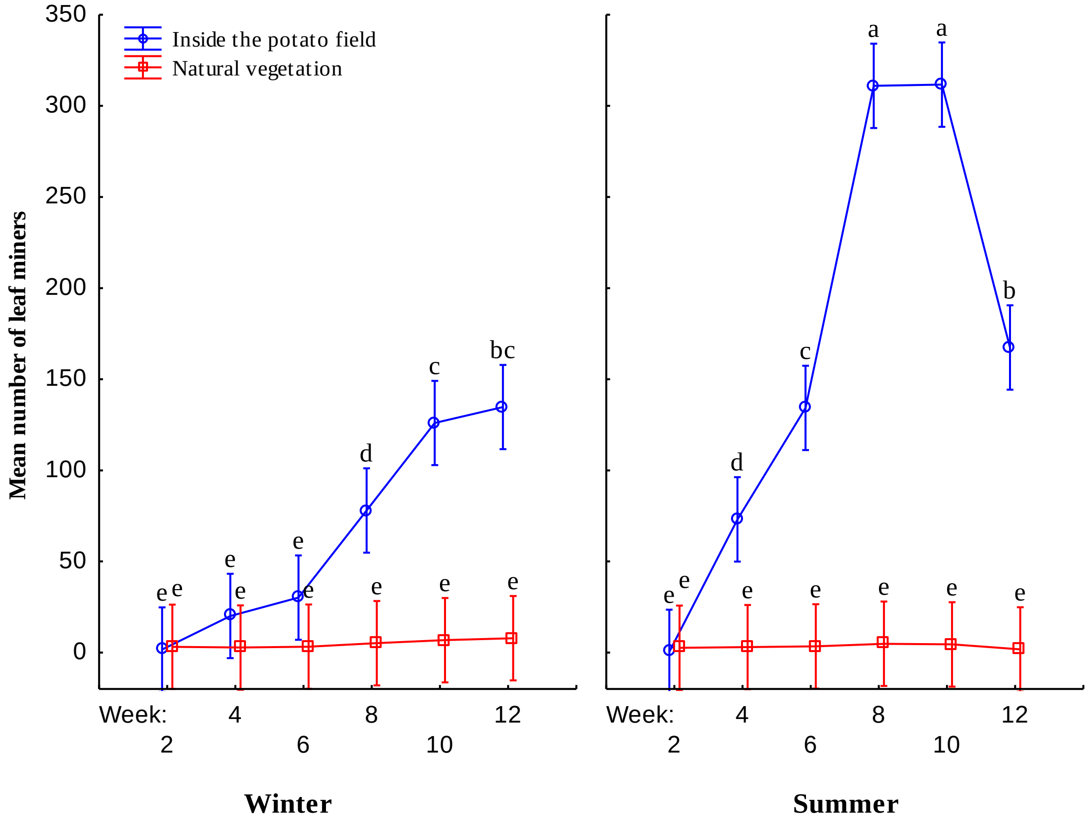

Fig. 2. 5.Mean number of adult leaf miners caught on yellow sticky traps, throughout two growing seasons (winter: summer) in two different potato fields and adjoining natural vegetation.

Damage assessments and persistence after harvest

The damage assessments demonstrated that in as early as week 4, about 5 % of the lower potato leaves had leaf miner larvae and punctures. The populations of the leaf miners on the lower leaves increased gradually in both the monitored fields. However, there was leaf miner presence on the lower leaves in week 2 during the winter monitoring period. The infestations increased with plant maturity. Therefore, seven weeks after plant emergence, 50 % of the plants monitored had leaf miner infestation (leaf miner punctures and leaf mines) and this was observed during both monitoring periods.

During the last 2 weeks of plant development (tuber maturity), severe damage was observed in both fields, amounting to 100 % leaf damage (Fig. 2.6). There was leaf miner damage caused by punctures and mines from as early as week 4 after plant emergence during winter and week 2 during the summer monitoring period.

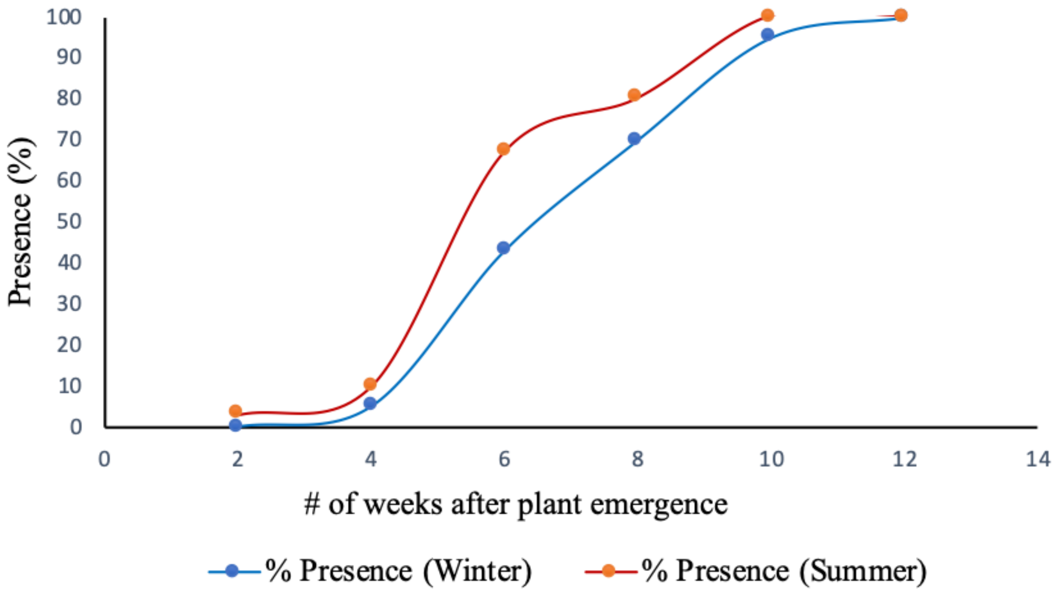

Fig. 2. 6. Leaf miner (*Liriomyza huidobrensis*) larvae and punctures found on the lower leaves during the survey of population dynamics over two seasons (winter and summer, year) in the Sandveld, Western Cape province, South Africa.

The sampling for persistence in the soil was only conducted once and as such there must be a possibility of the pupae to overwinter in the soil until a new or suitable crop is planted. However, these are just assumptions as this was a one-time sampling. The number of pupae found after the winter monitoring period reached an accumulated amount of 50. However, after keeping the pupae for more than 10 days at 25 ºC and long photo period (16 L: 8 D), they did not hatch into adult leaf miners, this could be associated with the pests overwintering strategy.

Insect rearing and parasitoid

For larval rearing, tomato and bean plants proved to be suitable hosts. While on weed plants, it was difficult to get a second-generation leaf miner, due to the lower number of punctures on the leaves. There was a reduction in the number of adults after each generation with all host plants. The recorded life cycle for three generations on bean plants was 22, 24 and 25 days, respectively. The larval stage took place six days after punctures were observed on the leaf surface. The larval stage usually lasted between 9 - 12 days depending on the generation. Pupation usually lasted for 7 days, and after adult emergence, the cycle began again for the next generation. In the laboratory three generations were completed from the original colony, thereafter it collapsed.

The parasitoid population obtained was relatively low both in numbers and diversity, which could be attributed to the time of the year when the experiment was conducted during the winter monitoring period. In total, 50 parasitoids emerged from the sampled leaves, most of which were identified as *D. isaea*. The parasitoid species that emerged from the monitored fields, even though they were low in quantities, are presented in Table 2.1. *Diglyphus isaea* was the only parasitoid species found in the Sandveld region.

Table 2. 1. Parasitoid species of *Liriomyza huidobrensis* found during the summer and winter monitoring period of the potato fields.

Parasitoid species	**Origin**
Diglyphus isaea	-32.588694,18.335528
Dacnusa sibirica	-32.588694,18.335528
Alysiinae sp.	-32.588679,18.335529
Eulophinae sp.	-32.588679,18.335529
Utetes africanus	-32.588679,18.335529

Alternative host survey

Several species of plants and weeds were identified as hosts, some of which were collected directly from the potato fields and others used to rear leaf miners in the laboratory (Table 2.2). Most host plants found during the monitoring were from the Amaranthaceae and Solanaceae family.

Table 2. 2. Alternative host plants of *Liriomyza huidobrensis* found Phillipi and Sandveld region of South Africa.

Scientific name	**Common name**	**Family**
Phaseolus vulgaris	Common bean	Fabaceae
Solanum lycopersicum	Tomatoes	Solanaceae
Chrysanthemum sp.	Chrysanthemum plants	Asteraceae
Alium cepa	Onion plants	Amaryllidaceae
Amaranthus sp.	Amaranthus	Amaranthaceae
Brassica campestris	Field mustard	Brassicaceae
Brassica oleracea	Wild cabbage	Brassicaceae

Chenopodium murale	Chenopodium plants	Amaranthaceae
Solanum nigrum	Black nightshade	Solanaceae
Solanum viarum	Tropical soda apple	Solanaceae
Conyza bonariensis	Hairy Fleabane	Asteraceae
Amaranthus sp.	Amaranthus plants	Amaranthaceae

Off season sampling of leaf miners in the natural vegetation

Leaf miner populations were low in the natural vegetation through the off-season monitoring period. The results showed that the mean trap catches of the leaf miners did not differ significantly ($F_{10, 130} = 0.803$, $P = 0.625$) between trappings (Fig. 2.7). However, there were significant differences ($P < 0.01$) observed in the population densities of leaf miners in surrounding natural vegetation between week 2, 4 and 8. There was a build-up in mean trap catches between week 4 and 8 which slowly dropped throughout the monitoring period. The highest number of flies were caught at week 8 (Fig. 2.6).

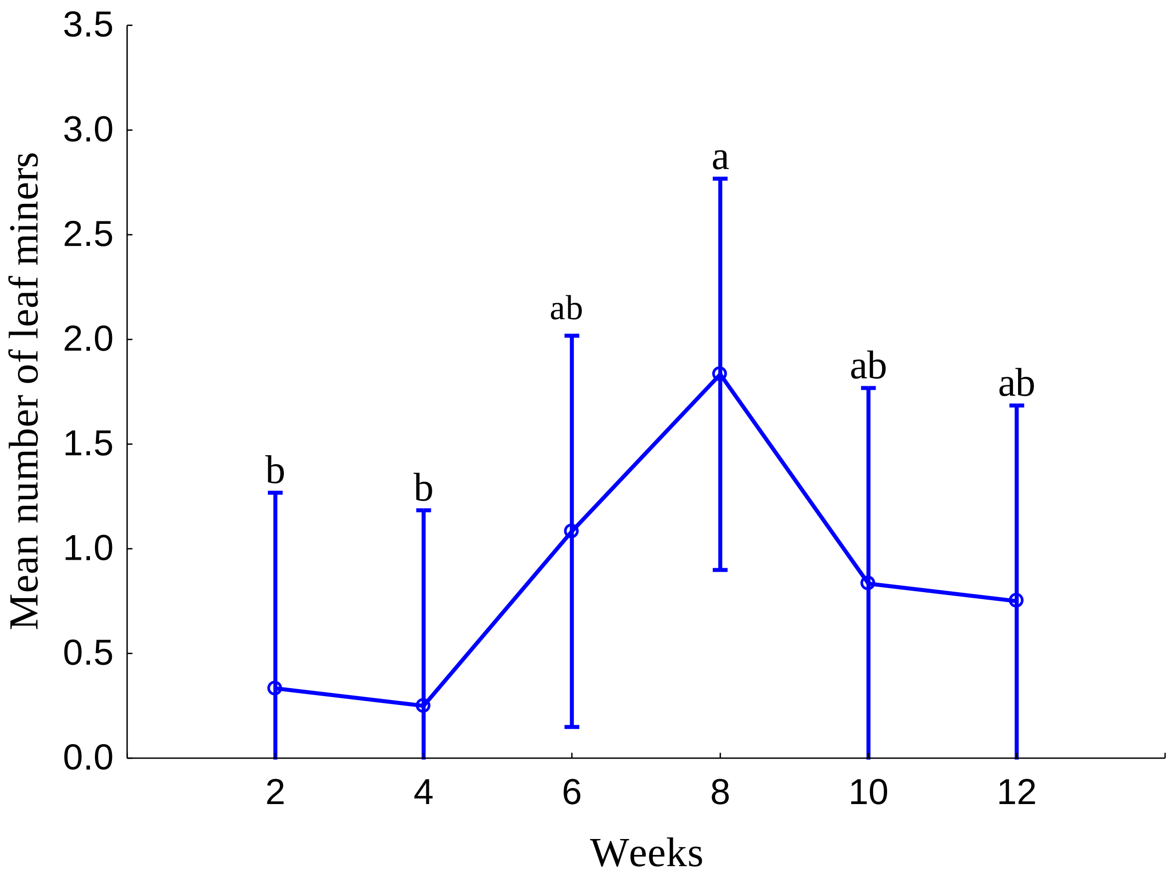

Fig. 2. 7. Adult *Liriomyza huidobrensis* found on yellow sticky traps during a survey of leaf miner abundance in the natural vegetation surrounding a potato field after harvest.

DISCUSSION

The potato leaf miner presents severe problems to potato growers during all phenological stages of the plant and causes problems at tuber maturation, as it reduces potato yield. This study is a preliminary investigation into establishing the mode of infestation of the leaf miner in the Sandveld area, in the Western Cape province of South Africa. During this study, population densities of the leaf miners slowly increased during early potato growth, but steadily increased from flowering onwards. Several studies found that the in-migrant adult populations initiate infestations in small potato plants at the beginning of the growing season (Mujica *et al.* 2000). However, as the plants developed, the number of adults caught on traps increased a week before flowering. Thus, the results from the present study are similar to those obtained by Vincini and Carmona (2006) regarding the dramatic increase in population density before flowering and decrease in population density during plant senescence. The results obtained in both seasons showed a significant association between *L. huidobrensis* populations and potato development, as also found by Lopéz *et al*. (2010). The monitoring of the first field (located in Saamstan) was in the winter period between June, July, August, and early September, while the second field (located in Saamstaan) was between October, November, December, and early January (summer period). This would explain the lower population densities found during the winter monitoring period and higher densities in summer. The population densities sampled in winter kept increasing until harvest, while during summer there was a reduction in the number of adults caught on traps at the beginning of plant senescence, similar results were obtained by Vincini & Carmona (2006) who concluded that *L. huidobrensis* is present throughout all potato development stages with a population decrease during senescense. Mean numbers of the leaf miners within the surrounding natural vegetation remained low throughout the two seasons, indicating that it is unlikely that this habitat type contributed to its spread to the potato field. The off-season monitoring showed very low numbers of adult leaf miners in the natural vegetation after potato harvest and as such this preliminary study only indicates the likelihood of leaf miners migrating from natural vegetation to new potato fields. However, this preliminary result does not have sufficient evidence to suggest that natural vegetation is used as a refuge for leaf miners when potatoes are not available, and further studies to broaden our knowledge on understanding the dynamics of the leaf miner should be conducted in this regard. However, a study conducted on *L. sativae* regarding the influence of temperature on flight potential showed that the flight ability of this species at temperatures between 18 °C and 36 °C was 8.22 km at a speed of 1.10 km/h (Zhongren *et al.* 2002).

The general tendency of the survey result is that the level of damage of the pest is slightly lower during winter and higher during summer. On average, the total leaf damage reached more than 50 % in as early as week 7, which coincided with the flowering period. This study confirmed a leaf damage level increase as the crop developed at all canopy layers both during the winter and summer period. The most severe damage was recorded on the lower growing leaves of a potato plant. However, as the crop produced new leaves, there were more opportunities for adult fly feeding, oviposition points, and larvae development areas, resulting in an increasing population. Similar results were obtained by Mujica & Cisneros (1997), recording a higher damage index on lower crop layer and lower damage on the intermediate and upper crop layer. The damage pattern is related to the egg extrusion phenomenon that is related to leaf age (López *et al.* 2010). Egg extrusion is referred to as the hypersensitive reaction that causes hypertrophic cell growth, that eventually leads to egg ejection (Facknath 2005). Egg extrusion is very high in young leaves and lower in older leaves because of the hypertrophic cell growth of new leaves, which leads to egg ejection and exposes the eggs to the environment and natural enemies (Facknath 2005; Videla & Valladares 2007). The pupaal persistence within the soil showed no adult emergence, and this could be associated with pupal mortality. Noujeim *et al.* (2013) suggested that the pupae could survive for two weeks at 4 ºC but after that, they start dying. Under other conditions the failure of pupae to emerging could be associated with their overwintering strategy, which could be as a result of changes in climate Tougeron *et al.* (2018) reported modifications of the structure and composition of insect communities, consequently affecting the functionality of organisms at very low temperatures.

Tomatoes and bean plants proved to be useful for larval development (rearing). However, the beans were better hosts in this regard when compared to tomato plants, likely due to their large leaf surface area, which permitted more punctures on one single leaf. Also bean plants are relatively fast-growing and only require 15 days before they become suitable for leaf miner production. The host plant plays a vital role in the fitness of larvae (Maharjan & Jung 2016). Having the right plant for insect rearing is, therefore, cardinal. Salvo & Valladares (2009) suggested that faba bean plants are suitable hosts, because it is easier to grow and has a large surface area for larvae to mine. Overall, there was also a considerable range of alternative hosts in vegetable fields from Philippi. Therefore, it is important to understand the dynamics of alternative hosts as well as their ecology.

In this study, the parasitoid *D. isaea* was the most abundant parasitoid species recorded even though there were other parasitoid species present. Other studies in Africa also reported the establishment of this parasitoid in southern Africa (Musundire *et al*. 2012; Chabi-Olaye *et al.* 2008). However, comprehensive studies on diversity and its potential to be mass-produced are needed. The parasitoid *Utetes africanus* (Wharton) (Hymenoptera: Braconidae) has no records of association to potato pests in the Western Cape province. However, its association with fruit feeding insects has been recorded especially on the olive fruit fly *Bactrocera oleae* (Rossi) (Tephritidae: Diptera) (Powell *et al*. 2019). *Dacnusa sibirica* (Telenga) (Hymenoptera: Braconidae) is the parasitic wasp used against leaf mining larvae (CABI 2019), and records of its use exist in South Africa on other leaf-mining insects through a product called Minusa from Koppert biological systems SA (Pty) Ltd. There are records of *Alysiinae* sp. in South Africa, even though there are no reports on its commercial use (Peris-felipo & Belokobylskij 2016). Furthermore, there have been records of the use of parasitoids from the Eulophidae family against the groundnut leaf miner pupae, *Aproaerema modicella* (Deventer) (Lepidoptera: Gelechiidae) (Van der Walt *et al.* 2009).

In conclusion, the data provided do not give conclusive analysis as to how potato fields are infested by leaf miners. The low leaf miner densities in the surrounding natural vegetation indicate that this is most likely not a major habitat for leaf miners in the Sandveld. More likely, the build-up within the fields later in the season is due to the abundant food found within the fields. More studies need to be conducted to validate the mode of infestation in different environmental conditions and different vegetation types. The present study also confirmed that *L. huidobrensis* is present at all developmental stages of the potato plant, with a steady increase as the plants mature, with a peak during flowering. Lastly, there are several plant hosts for this pest, thus, the need for control measures of various weed species, especially those that do not harbour beneficial insects.

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CHAPTER 3

Isolation of Entomopathogenic Nematodes and Fungi for the Control of the Potato Leaf miner, *Liriomyza huidobrensis* (Diptera: Agromyzidae)

ABSTRACT

Entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF) are some of the most common biological control agents surveyed worldwide. The use of local entomopathogens in the control of insect pests has widely been encouraged for many years. The potato leaf miner, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae), is a pest of economic importance of potatoes across the world. In South Africa, the first reports of the leaf-mining larvae were made in 2000. In this study the occurrence of entomopathogens was assessed in soil samples collected in potato fields from the Sandveld region in the Western Cape province. A total of 160 sub-samples were collected from different potato fields. EPNs were isolated from only one site out of the 16 fields (6.25 %) sampled and EPF from five sites (31.25 %) using *Galleria mellonella* (Lepidoptera: Pyralidae) (wax moth) larvae a trap insect. An unknown *Acrobeloides* species, a bacterial-feeding insect associated nematodes, was trapped from two fields (12.5 %). Overall, eight fields were positive for both EPNs and EPF. All isolates were identified to species level, using molecular techniques. The screening of different *Metarhizium robertsii* isolates against the pupal stage of *Liriomyza huidobrensis* showed that the highest percentage of mortality was obtained from the *M. robertsii* isolate 10C (90.63 % ± 5.98 %) and overall, all fungal isolates performed well with mortalities ranging between 80 % and 90 %. The phylogenetic analysis of the internal transcribed spacer (ITS) region identifed the entomopathogenic nematode to be *Heterorhabditis zealandica*. EPF have shown biocontrol potential as a soil application against *L. huidobrensis* pupae.

Key words: *Acrobeloides*, entomopathogenic fungi, entomopathogenic nematode, *Heterorhabditis zealandica*, screening

INTRODUCTION

The potato leaf miner, *Liriomyza huidobrensis* (Diptera: Agromyzidae), is a pest of economic importance of potatoes across the world, including South Africa, and is found in all potato growing areas (Mujica & Kroschel 2013). It originates from Central and South America where it was reported prior to 1980 (Lanzoni *et al.* 2009). In South Africa, the presence of *L. huidobrensis* on potatoes was first recorded in 2000 (Picker *et al.* 2002; Visser 2009; Weintraub *et al*. 2017).

The life cycle of *L. huidobrensis* is composed of four stages; first, a female can lay up to 400 eggs. The developmental period for eggs lasts between two and five days before hatching (Visser 2009; CABI 2018). The larval stage undergoes three instars, the first instar being colourless on hatching and last instar larvae are pale yellow-orange (Weintraub & Horowitz 1995). After larval development, the last instar larvae make a slit on the leaf surface to leave the mine to pupate (Visser 2009). The pupae, brown in colour, in most cases fall to the ground (Visser 2009). After 10 days, a small greyish-black adult emerges and will begin to feed on the leaf surfaces (Weintraub *et al*. 2017).

The potato leaf miner is a polyphagous insect that affects several horticultural crops and associated weeds (Mujica & Cisneros 1997). The damage by adult females is caused by ovipositor probing and feeding, while the larval damage causes mines within the leaf mesophyll, which ultimately results in tissue damage (López *et al.* 2010). To date, chemical insecticides have been the common control strategy of the potato leaf miner (Rondon 2010; Mujica & Kroschel 2013). This can be attributed to the fact that synthetic insecticides are a straightforward way to decrease insect population densities before reaching the economic damage threshold (Visser 2005; Okoth *et al*. 2014; CABI 2018). However, insect resistance to certain chemical insecticides has been reported, due to pesticide overuse (Okoth *et al.* 2014; Weintraub *et al.* 2017). These insecticides do not only contribute to pesticide resistance, but also cause natural enemy reduction (Okoth *et al*. 2014).

To avoid control strategies that rely solely on chemical insecticides, several biological control strategies have been studied (Gangwar 2017). Entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPNs) are promising biocontrol agents against several insect species (Chandler *et al.*1997; Stock & Goodrich-Blair 2012). EPF have biocontrol potential against a wide range of arthropods and are cardinal elements in agricultural systems (Quesada-

Moraga *et al.* 2006). The success of EPF is dependent on the ability of the fungi to invade and penetrate the host through the insect cuticle, which protects insects against several external threats (Inbar & Gerling 2008). Both mechanical pressure in the germinating tube and the action of degradative enzymes result in EPF penetration through the insect cuticle (Bonnie *et al.* 2004; Zimmermann 2007). Thereafter, fungal hyphae develop inside the insect, which later invade the tissues and organs resulting in insect mortality (Mantzoukas & Eliopoulos 2020). Following insect mortality, the fungus produces new spores on the cadaver. The period of insect mortality caused by EPF infection is between (3 and 7 days). However, this is dependent on the type of fungus and the number of spores used (Mantzoukas & Eliopoulos 2020). Some studies demonstrated EPF to be effective against dipteran leaf miners (Migiro *et al.* 2010). However, most of these studies are limited to using different fungal isolates against the pupae stage and not the adults because field application of fungi on adults is diffict (Borisov and Ushchekov 1997; Migiro *et al*. 2010).

Another promising biocontrol agent against dipteran insects are EPNs (Jacob & Mathew 2016). The insect parasitic nematodes or EPNs occur naturally in the soils across the world and are natural enemies of different insect species (Stock & Goodrich-Blair 2012; Lu *et al.* 2016). However, only a few species from the genera *Heterorhabditis* and *Steinernema* demonstrated the potential to infect leaf miners (Liu *et al.* 2009). The EPNs are associated with the symbiotic bacteria, *Xenorhabdus* (Enterobacteriales: Enterobacteriaceae) (Stock & Goodrich-Blair 2012). The mode of infestation includes the penetration of the infective juveniles (IJs), which later release symbiotic bacteria after penetrating the haemocoel of the host insect, thus, cause mortality within a 48-hour period (Hazir *et al.* 2004). The efficiency of the nematode is dependent on the number of nematodes that penetrated and the size of the target insect (Kaya & Gaugler 1993; Dillman & Sternberg 2012).

The potential for EPNs and EPF as potential biological control agents of different insect species has been widely investigated and several species have been commercialized worldwide (Abate *et al.* 2017) and both these entomopathogens have the potential to be included in integrated pest management programs (IPM), together with other pest control strategies.

The aims of this study were to survey potato fields infested with *L. huidobrensis*, for the presence of locally occurring entomopathogens and to test the virulence of these isolates against the pupal stage of *L. huidobrensis*. This is a first step in developing a locally adapted biological control programme against *L. huidobrensis*.

MATERIALS AND METHODS

Source and collection of soil samples

A total of 32 soil samples, comprised of 160 sub-samples, were collected from 16 different potato growing fields in the Sandveld, Western Cape province, South Africa (Fig. 3.1) & Table 3.1. The soil samples were collected from five different areas within each site (field) and mixed to form a single soil sample. This was done by removing the top soil with any vegetation/leaf litter, and then using a shovel to collect the sandy soils at a depth of between 15 and 20 cm. Eighty of these sub-samples were used for the survey of the EPNs, while the other 80 sub-samples were used for the EPF survey. The soils were labelled and carried in transparent plastic bags to the laboratory and all the soil samples were collected from potato fields which were in production, and the management of these fields were at the grower's discretion.

Table 3. 1. Sampling sites and vegetation from which the soil samples for the survey of entomopathogens in the Sandveld region of the Western Cape province, South Africa.

Site number	Site Location	Vegetation
S1	32°35'19.3"S 18°20'07.9"E	Mature potato plants
S2	32°34'50.8"S 18°20'05.4"E	Newly planted potatoes
S3	32°34'23.8"S 18°22'47.1"E	Empty potato field (ploughed)
S4	32°33'18.3"S 18°24'38.3"E	Mature potato plants
S5.	32°33'50.6"S 18° 24'25.9"E	Mature potato plants
S6.	32° 33'47.3"S 18°25'39.6"E	Mature potato plants
S7	32°33'32.7"S 18°25'59.0"E	Mature potato plants
S8	32°34'56.0"S 18°22'35.3"E	Newly planted potatoes
S9	32°37'54.9"S 18°23'54.3"E	Newly planted potatoes
S10	32°39'19.3"S 18°23'56.2"E	Mature potato plants
S11	32°40'58.0"S 18°41'12.4"E	Newly planted potatoes
S12	32°40'22.9"S 18°41'57.0"E	Newly planted potatoes
S13	32°40'26.4"S 18°42'09.9"E	Mature potato plants
S14	32°40'43.0"S 18°41'55.6"E	Harvested potato field
S15	32°40'10.4"S 18°40'47.2"E	Empty potato field (ploughed)
S16	32°34'01.1"S 18°21'02.4"E	Empty potato field (ploughed)

Fig. 3. 1. Map indicating soil sampling sites in potato fields in the Sandveld region of the Western Cape.

Isolation of EPNs and EPF

The soil samples were transferred to 1-L plastic containers, then mixed and sieved to remove large particles like rocks and leaf debris. A total number of 10 *Galleria mellonella* (Lepidoptera: Pyralidae) (wax moth) larvae were used as bait insects for each soil sample in the plastic containers and for aeration the 1-L plastic containers were not completely filled, but a 10 cm breathing space was left. If any of the soils were dry, distilled water was used to moisten them to maintain humidity during the baiting process (Meyling & Eilenberg 2007; Goble 2009). The 1-L plastic containers were kept in a room with a controlled temperature of ± 25 ºC (Meyling & Eilenberg 2007). Mortality of the *G. mellonella* larvae was observed daily for two weeks.

Isolation and identification of nematodes

The dead larvae were collected from the soil in plastic containers daily and rinsed in sterile water or with a sodium hypochlorite solution (1 %). Following this process cadavers were incubated in Petri dishes at 25 ºC for two to three days, then transferred to modified White traps for nematode offspring recovery (White, 1927). The nematode isolates were kept in vented culture flasks in 150 ml of distilled water within the Nematology laboratory at Stellenbosch University and incubated at 14 °C. The culture flasks were shaken every two weeks to avoid nematode clumping and to promote ventilation. The infective juveniles (IJs) were kept by recycling through wax moth larvae every three months. DNA extraction involved the use of the first-generation female in a lysis buffer of 30 µl (buffer, DNA free water, and proteinase K). The EPNs were cut on the side of a 0.5 l Eppendorf tube using a syringe needle and immediately put on ice. The tubes were then put in a - 80 ºC freezer for 30 min. After that, the supernatant with the DNA of the nematodes was used for PCR. In short, the PCR of the transcribed spacer ITS (ITS1-5.8S-ITS2) region of the smaller subunit of the 18S rRNA gene was as follows; a primary mix having DNA free water, buffer, the primers (TW81: 5´-GTTTCCGTAGGTGAACCTGC3´(forward) and AB28: 5´-ATATGCTTTCCGTA GGTGAACCTGC 3´ (reverse) (Hominick *et al.* 1997), the mixture was prepared in an previously autoclaved 2 ml Eppendorf tube and homogenized in a vortex mixer. Thereafter, 20 µl of the primary mix was pipetted into autoclaved Eppendorf PCR tubes and 5 µl of nematode DNA was added. The Eppendorf tubes were put in a thermocycler with 36 cycles at 94 °C in 10 min, 94 °C at 30 °C, 55 °C at 30 °C, 72 °C at 1 min, 72 °C at 7 min, and 4°C. The PCR samples were then run on gel electrophoresis and viewed under ultraviolet light. Samples with visible bands were selected and their PCR products were sequenced at the central analytical facility (CAF) at Stellenbosch University. The sequences were aligned and edited using the software CLC Main Workbench and later NCBI database was used to verify the species.

Extraction and identification of EPF

Dead wax moth larvae (hard or showing overt mycosis) were washed using distilled water to remove any soils found on them and were then dipped in 70 % ethanol and rinsed with distilled water thereafter. The collected dead insects were plated on Petri dishes, then sealed with parafilm, placed in a 2-L container with paper towels, moistened with distilled water, and later kept incubated at 25 °C. Every week, the dead mycosed insects were collected, the fungi from the bodies were scrapped and placed on Saboraud Dextrose Agar with yeast (SDAY) plates. The fungi cultures were incubated at 25 °C in growth chambers and checked for fungal growth every four days and promising fungal isolates were grouped according to morphological characteristics. After sporulation of the fungal colonies on SDAY, a small sample from the growing colony was transferred onto a glass slide and then examined using a microscope for vegetative and reproductive characteristics such as the structures of the hyphae and the shape and size of the conidia as well as conidiophores. The extraction of the DNA was from six isolates that were selected morphologically. DNA extraction was done using Zymo Research Quick-DNA fungal/bacterial miniprep kit (Zymo Research Corporation), the protocol followed was the one supplied by the manufacturer. After the process of DNA extraction, the extracted DNA then passed through the process of PCR. The following primers were used, to amplify the internal and the translation elongation factor-1α protein coding gene (EF) sequencing primers; EF1F forward primer (5'-GTCGGTGGTATCGACAAGCGT-3')] and EF2R [reverse primer (5'-AGCATGTTGTCGCCGTTGAAG-3'). Ultimately, the un-purified DNA was sent for clean-up and sequencing at CAF, Stellenbosch University. The sequences were verified and edited using the software CLC DNA Workbench.

Screening of different Metarhizium robertsii_isolates

Pupae of *L. huidobrensis* were used in the assessment to test the virulence of five different *Metarhizium robertsii* Bisch., Rehner & Humber isolates following the same procedure as Noujeim *et al.* (2015). All the isolates were tested at 1×10^7 conidia/ml in sterile distilled water and 0.05 % Tween 20, while the control was treated with sterile distilled water containing 0.05 % Tween 20. A total of 192 pupae were used to test the virulence of all the *M. robertsii* isolates using 24-well bioassay plates as the test arena. The 24-bioassay plates were fitted with filter papers and alternate wells and eight pupae were used per treatment. The 24-bioassay plates were placed in 2-L plastic containers and incubated at 25 °C ± 2 °C in a growth chamber. Pupae mortality was recorded 7 days after treatment with the five fungal isolates. To confirm whether the pupae died due to fungal isolate exposure, the pupae were placed on SDA plates post surface sterilization and later incubated 25 °C ± 2 °C (Coombes 2012). Visual mycosis and fungal growth confirmed the cause of mortality.

Data analyses

To evaluate efficacy of the *Metarhizium robertsii* isolates, the collected data were analysed using the statistical software, STATISTICA 13.3 (TIBCO Soft Inc. 2016). Data were analysed using a one-way ANOVA (analysis of variance) & (VEPAC) comparison of means of mortality of different isolates.

RESULTS

Identification of entomopathogens

The EPF and EPNs isolated from this survey are listed in Table 3.1. EPNs were isolated from one field (6.25 %) and samples from five out of 16 fields (31.25 %) produced EPF isolates. The EPN species were isolated from field number 9 and the isolated species were molecularly identified as *Heterorhabditis zealandica* Poinar. The EPF isolated were *M. robertsii* and *Metarhizium majus* (Johnst.) Bisch. Molecular identification of EPNs was based on the ITS region. The EF protein coding gene was used in the identification of the fungi isolates to species level. The four sequences of the *H. zealandica,* the two sequences of insect associated nematodes *Acrobeloides* sp. and the five EPF isolates of *M. robertsii* and one *M. majus* were deposited in GenBank (Table 3.1).

Table 3. 2. Insect-associated nematodes and entomopathogenic fungi isolated from potato field soil samples in the Sandveld region of the Western Cape province, South Africa.

Isolations	Isolate Number	Region/ gene	Sequence length (bp)	GenBank Number ITS	GenBank Number EF	Origin
Heterorhabditis zealandica	S9	ITS	803	MW085080	n/a	-32.631928, 18.398416
H. zealandica	S9	ITS	800	MW085081	n/a	-32.631928, 18.398416
H. zealandica	S9	ITS	800	MW085082	n/a	-32.631928, 18.398416
H. zealandica	S9	IS	801	MW085083	n/a	-32.631928, 18.398416
Acrobeloides sp.	S4	ITS	751	MW150803	n/a	-32.563138, 18.427660
Acrobeloides sp.	S6	ITS	747	MW150804	n/a	-32.579270, 18.380687
Metarhizium robertsii	14N	EF	Pending	-	Pending	-32.678611, 18.698789
M. robertsii	13C	EF	Pending	-	Pending	-32.655359, 18.398941
M. robertsii	10C	EF	Pending	-	Pending	-32.678611, 18.698789
M. robertsii	14G	EF	Pending	-	Pending	-32.678611, 18.698789
M. robertsii	14	EF	Pending	-	Pending	-32.580764, 18.334819
Metarhizium majus	2G	EF	226	-	MW122513	-32.580764, 18.334819

The tree with the highest likelihood for the ITS gene region is shown below (Fig. 3.2). There were no phylogeny differences observed between the *H. zealandica* MW085080 sequenced isolate, and those from different parts of South Africa and Florida that were downloaded from the NCBI GenBank database for the phylogenetic tree based on the ITS region. *Heterorhabditis zealandica* did not show any differences in identity, but rather showed a 100 % similarity with GenBank deposited species from Florida. *Oscheius tipulae* was used as the out-group taxa.

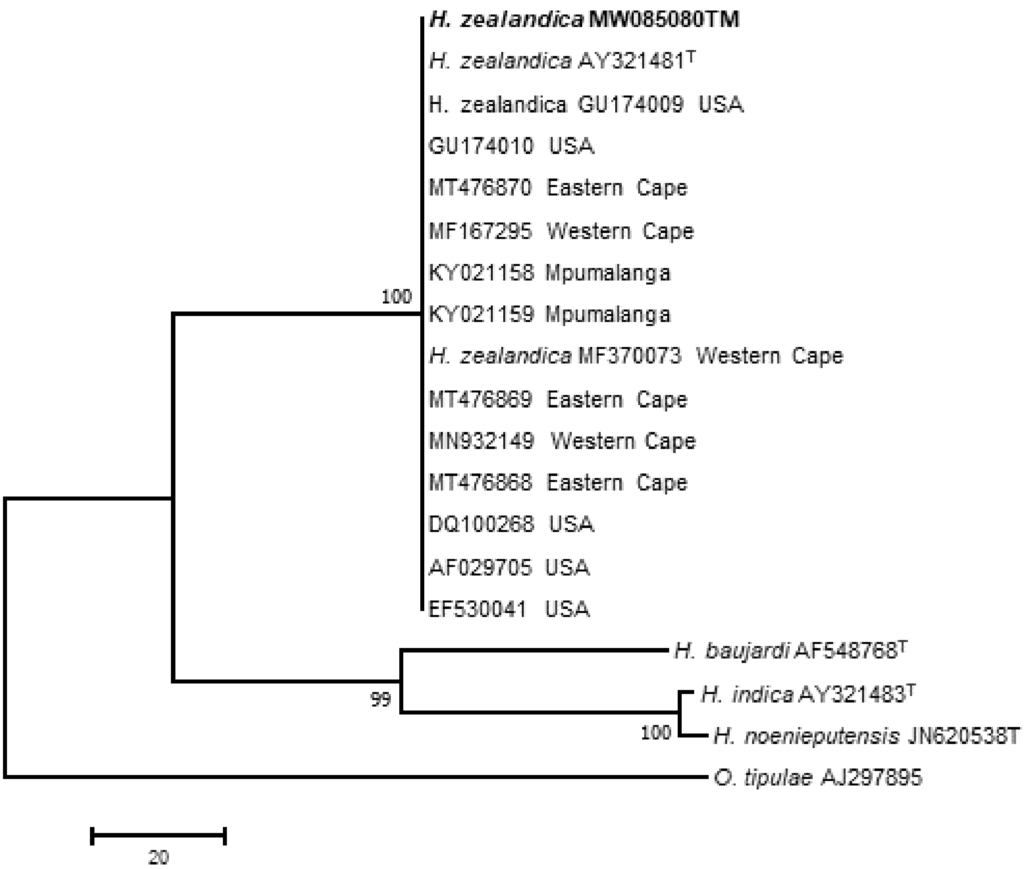

Fig. 3. 2. A phylogenetic tree based on the internal transcribed spacer (ITS) region, showing the relationships among some *Heterorhabditis zealandica* isolates, *Oscheius tipulae* was used as the out-group taxa.

Screening of Metarhizium robertsii isolates

The results obtained from screening five different *M. robertsii* isolates at a 1×10^7 conidia/ml showed significant differences (ANOVA, $F_{10, 34} = 5.951$; $p < 0.001$) in the percentage mortalityof pupae. The highest percentage mortality was obtained from the *M. robertsii* isolate 10C (90.63 % ± 5.98 %), followed by *M. robertsii* isolate 14N (87.50 % ± 8.84 %), *M. robertsii* isolate 13C (84.38 % ± 5.98 %), *M. robertsii* isolate 14G (81.25 % ± 8.09 %) and *M. robertsii* isolate 14! (81.25 % ± 8.09 %), with no significant difference between each other ($p = 0.876$). However, the percentage mortality of pupae caused by all the *M. robertsii* isolates differed significantly ($p < 0.05$) from the pupae mortality of the control treatment (3.13 % ± 3.13 %) (Fig. 3.3).

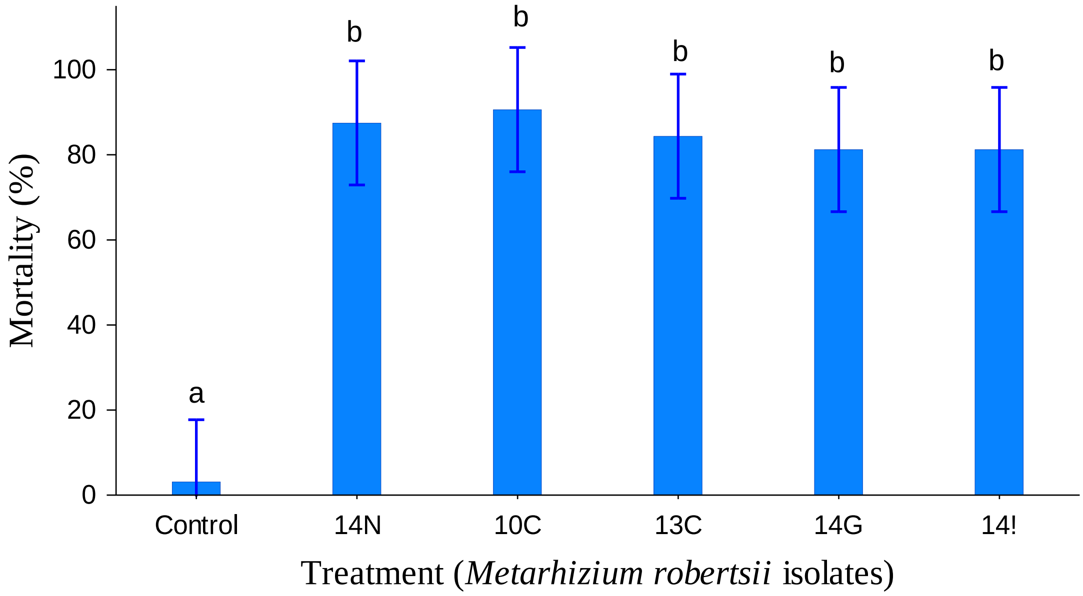

Fig. 3. 3. Average percentage mortality (95 % confidence interval) of the pupae of *Liriomyza huidobrensis*, as induced by five different *Metarhizium robertsii* isolates, and the control treatment using distilled water only, 7 days post-treatment (one-way ANOVA, $F_{10, 34} = 5.951$; $p < 0.001$). Different letters on the bars indicate significant difference ($p < 0.05$) between the treatments and the mortality of *L. huidobrensis* pupae.

DISCUSSION

Studies have demonstrated the potential of EPF and EPNs as biocontrol agents for many insect pests (Chandler *et al.* 1997). Nevertheless, developing them as commercial insecticides involves many steps, including isolation from either the environment, or diseased insects, the selection of the best strain through virulence and pathogenicity assays and storage properties (Almanoufi *et al.* 2012). Factors such as high virulence will permit the inclusion of both EPF and EPNs in integrated pest management. In this study, we demonstrated the natural occurrence of EPF and one EPN species through a specific survey of the Sandveld region in different potato fields in summer. Local EPNs have shown potential to control leaf mining insects, for example, Steyn *et al*. (2019) showed virulence of *H. zealandica* even though the percentage mortalities were very low against the larval stage of the lepidopteran leaf miner. Thus, the presence of this EPN species in potato fields is promising and is a step closer to developing an efficient management practice. There was little diversity in EPF species in the Sandveld region with *M. robertsii* and *M. majus* being the only two species found. The results are in concordance with those obtained by Barker *et al*. (1998) who concluded that cultivated areas are likely to exhibit less diversity with regards to entomopathogens than undisturbed areas. In this study, all the entomopathogens were isolated from soil samples. Abaajeh & Nchu (2015) also successfully isolated six different EPF from soil samples and one EPN isolate using the same baiting technique in the study conducted in South Africa.

The use of ITS gene regions enable the molecular identification of the collected nematode species, while the EF region was used for the identification of fungal isolates by means of amplification. The phylogenetic relationships among the collected *H. zealandica* and some that were collected within South Africa and the United States showed 100 % similarity. Overall, the fungi isolates found, *M. robertsii* and *M. majus* are known to cause virulence within a wide range of insects (Meyling & Eilenberg 2007). This would be the second report for *M. majus* from South African soil. The first report was from an organic apricot orchard on the farm Tierhoek, near Robertson in the Western Cape province (Mathulwe *et al*. 2020). The isolation of *M. majus* from potato soil indicates the presence of another potential species of EPF that can be tested as a biocontrol agent against *L. huidobrensis*. Studies in Japan have indicated *M. majus* to be effective against Coleoptera (Nishi *et al.* 2015).

Studies show that after *Liriomyza* pupae leave the mine, they fall to the ground and as such become vulnerable to different predators (Parella 1987; Wei et *al.* 2000; Visser 2009). Therefore, using soil dwelling entomopathogens like EPNs and EPF is a better alternative in the management of the pupae stage of the potato leaf miner (Visser 2009; Gathage *et al.* 2016; Jacob & Mathew 2016). Also controlling the leaf miner in the pupae stage has a greater impact on the emerging adults. The emerging adults infected with fungi will lay fewer eggs and have a shorter life cycle (Migiro *et al.* 2011; Akutse *et al.* 2013). The possibility of using EPNs and EPF to control the pupae stage of the potato leaf miner will allow optimum suppression of pupae as they are vulnerable and immobile, therefore, field applications should be done before planting.

An insect associated nematode, *Acrobeloides* sp., was also molecularly identified using the ITS region. There are few identified species from the *Acrobeloides* genus and as such there was no phylogenetic tree made for a definite comparison. The nematode species belonging to the genus *Acrobeloides* are not classified as EPN, but rather as an insect-associated nematode, which is a non-pathogenic bacterivore. It is very common in sandy soils that are subjected to high temperatures and coastal regions (Baquiran *et al.* 2013), thus explaining its presence in the Sandveld region which is a sandy area and near the coast.

Screening and determining the differences in virulence levels of the five *M. robertsii* isolates against the pupal stage of *L. huidobrensis* were important steps in developing an integrated management programme. Studies have demonstrated differences in pathogenicity of different isolates of the same fungal species (Migiro *et al.* 2010). Although all five *M. robertsii* isolates tested, in the current study were pathogenic, they did no differ significantly ($p > 0.05$) in their pathogenicity potential. In a similar study, where different isolates of *M. anisopliae* were evaluated against the adult stage of *L. huidobrensis*, the results indicated differences in virulence levels ranging between 45.3 % to 98.7 % (Migiro *et al.* 2010).

Heterorhabditis zealandica has shown potential in the control of *Cydia pomonella* (codling moth) (De Waal *et al*. 2013), *Phlyctinus callosus* (banded fruit weevil) (Ferreira *et al*. 2014) and *Planococcus citri* (citrus mealybug) (Van Niekerk *et al*. 2012). Therefore, it is important that this nematode species be tested against *L. huidobrensis* in future studies. This isolate of *H. zealandica* was associated with bacteria that turn the host greenish in colour. In other studies, the bacteria of *H. zealandica* turned the host steel grey and the bacteria was described as a new species for South Africa, *Photorhabdus zealandica* (Ferreira *et al.* 2014). However, James *et al*. (2018) found *H. zealandica* with bacteria that also turned the host species greenish, while Steyn *et al*. (2020) conducted a survey of *H. zealandica* and recorded instances where the host turned red as well as greenish. It can be assumed that the South African *H. zealandica* is associated with three different mutualistic bacteria and needs further investigation as to their efficacy against *L. huidobrensis* and other economically important pests.

These results provide an initial insight into the diversity of EPNs and EPF in the Sandveld in16 potato fields. The analysis of phylogenetic relationships has provided us with information on how rare *H. zealandica* is in other countries, albeit strong similarities between local isolates and those from the United States exist. On the other hand, the results obtained will allow further analysis of the effect of both EPF and EPNs to different life stages of *L. huidobrensis*. The *M. robertsii* isolates, in particular, indicated a relatively high level of virulence, which have the potential for being a successful biocontrol agent in the management of *L. huidobrensis*. Future use of both EPN and EPF in combination, may show a synergistic effect, especially on the pupae and the emerging adult (both being in contact with the soil) and offer a long window (± 10 days) of opportunity for infection with these biologicals.

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CHAPTER 4

Laboratory Screening of South African Entomopathogens for control of the Potato Leaf Miner, *Liriomyza huidobrensis*

ABSTRACT

Liriomyza huidobrensis Blanchard (Diptera:Agromyzidae), commonly known as the potato leaf miner, is an important pest of potatoes in the Western Cape province of South Africa. Chemical insecticides have played a vital role in reducing *Liriomyza* populations in most potato fields. However, there are growing concerns around the risks associated with insecticide resistance to chemicals and natural enemy reduction. This has prompted the search for alternative control approaches for the leaf miner. Some of the most successful alternative approaches are biocontrol agents such as entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPFs). In this study, EPNs and two EPFs have been evaluated against the larval and pupal stages of *L. huidobrensis* under laboratory conditions. The mortality (by infection) of the entomopathogens was investigated, of which *Heterorhabditis baujardi* was the best performing treatment (> 55 % mortality) for the pupae and larvae stages *L. huidobrensis*. In the case of EPF, *Metarhizium robertsii* outperformed *Beauveria bassiana* with more than 80 % mortality.

Key words: *Beauveria bassiana*, Entomopathogenic fungi, Entomopathogenic nematodes, *Liriomyza huidobrensis*, *Metarhizium robertsii*,

INTRODUCTION

Potato, *Solanum tuberosum* L. (Solanales: Solanaceae), is an economically important crop consumed worldwide (Caldiz *et al.* 2009). They originate from the tropical and subtropical regions of America (Spooner *et al.* 2005) growing on all continents, except Antarctica. Potatoes are considered the third most vital crop in the world, following cereals (Lutaladio & Castaldi 2009). In recent years, developing countries have steadily increased the production of potatoes (NAMC & Commark Trust 2006; Lutaladio & Castaldi 2009). Currently, it is regarded as a staple food crop in most areas, which can meet the demands of the growing human populations (Birch *et al.* 2012). However, there are several reports on the increase of insect pests and diseases in most potato growing areas (Merz & Falloon 2009). As a result, meeting the food demand in a steadily increasing global population remains one of the major emerging challenges. The global population will rise significantly by the year 2050, reaching more than 9.1 billion, and with this increase comes an increase in food demand (Godfray *et al.* 2010). Hence, the need for an extra 70 % in food production (Godfray *et al.* 2010; Hodges *et al.* 2011; Parfitt *et al.* 2010).

Potato is one of the most susceptible crops to insects and diseases found in arable rotations (Wright *et al.* 2017). Potato is usually affected by several pests, including insects like aphids, leaf miners and beetles, nematodes such as *Globodera* spp. (potato cyst nematodes), viruses and diseases such as *Alternaria* spp., *Phytophthora infestans* and *Rhizoctonia solani* (Wale *et al*. 2008). Thus, the primary key in crop protection is a practical approach to pest and disease management (Pawelzik & Möller 2014). Current management practices involve the use of synthetically produced active compounds (Murphy 2007). There are several insect pests attacking potatoes, however, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) and *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) are amongst the most important, causing significant crop damage, reaching up to 70 % yield reduction in both commercial and subsistence settings (Picker *et al.* 2002; Visser 2005; Rondon 2010; Mujica & Kroschel 2013).

Liriomyza huidobrensis (Blanchard) (Diptera: Agromyzidae), also known as the potato leaf miner, is a highly polyphagous pest causing substantial damage in potatoes and other vegetable crops (Weintraub *et al.* 2017; CABI 2018). Adult feeding and oviposition results in potato damage, and additionally the mining larvae feed on the leaf mesophyll (Chabi-Olaye *et al.* 2008). Pathogens may enter as a result of the punctures to be indirect damage, such as in the case of *Alternaria alternaria* (Fr.) Keissl, being the most common pathogen (Deadman *et al.* 2000). The lower leaves of potatoes are usually the first to exhibit infestation symptoms (Visser 2005). The severity of larval damage depends on crop development, with older and fully-grown plants being the most damaged, whilst the vegetative stages are less affected by damage (Visser 2005, 2009; Mujica 2016; CABI 2018).

The potato leaf miner can cause a reduction in production and is present in all potato crop development stages (Visser 2005; CABI 2018). Synthetic insecticides are used by farmers after noticing the first symptoms, to decrease insect populations below damaging levels (Okoth *et al.* 2014; Gangwar 2017). However, the constant use of these synthetic insecticides induces pest resistance, raises production costs, contaminate the environment, and reduces natural enemies (Okoth *et al.* 2014). Insect resistance on several *Liriomyza* species to certain synthetic insecticides is a common problem in many countries, even though diverse leaf miner populations may have different resistance profiles (Weintraub *et al*. 2017).

Synthetic insecticide resistance by pests such as the potato leaf miner, the destructive effects on the environment and the safety of humans have resulted in the development of biological control agents as alternatives in the management of insect pests (Inglis *et al.* 2001). Several microorganisms, including viruses, bacteria, protozoa, and fungi (Gangwar, 2017), are now common biocontrol agents of different insect pests (Quesada-Moraga *et al.* 2006). Preferred biological control options, such as entomopathogenic fungi, parasitoids, nematodes, and bacteria, are steadily increasing (Chandler *et al*. 1997; Stock & Goodrich-Blair 2012). Their efficacy in control of various insect pests has helped in the development of microbial insecticides, and the success of these control options reduce the need for synthetic pesticides (Bhattacharya *et al*. 2003; Hassan *et al.* 2016). However, in many cases, biological control agents should not be a stand-alone option, but should be integrated into a pest management system to use fewer chemicals, delay resistance, and prolong the effectiveness of these chemicals (Abate *et al.* 2017).

In recent years, the area of interest in several studies is developing biocontrol agents against different pests (Butt *et al.* 2001). Among this diverse group of biocontrol options stand entomopathogenic nematodes (EPNs), which deliver a natural ecological service by preventing insect population flare ups and offer a return to balance (Navon & Ascher 2000; Platt *et al.* 2019). Their broad host range and benign effect on the environment are some of the advantages that make nematodes effective against several insect pests (Kerry & Hominick 2002). There are a few species from the genera *Heterorhabditis* and *Steinernema* that showed the ability to infect leaf miners (Liu *et al*. 2009). Several studies have demonstrated potential in using EPNs for above ground insect pests including leaf miner insects and express the possibility of foliar applications, as the nematodes can enter the blotch mine on leaves to infect the feeding larvae (Harris *et al*. 1990; Platt *et al.* 2019a, b; 2020; Steyn *et al.* 2019).

The entomopathogenic fungi (EPF) of the order Hypocreales, especially *Beauveria bassiana* (Bals. -Criv.) Vuill. (Cordypitaceae) and species of the *Metarhizium anisopliae* (Metchnikoff) Sorokin (Clavipitaceae) complex, are among the world's most investigated biocontrol agents, and their capabilities showed great success (Erler & Ates 2015). Migiro *et al.* (2010) recently demonstrated the potential of EPF as biological control agents of dipteran flies. The mode of infestation of EPFs involves invading their hosts through the cuticle and would, therefore, be a great alternative for controlling several insect pests (Poprawski *et al.* 2000; Inbar & Gerling 2008). When using EPFs against crop pests, local or indigenous strains of EPF are preferred, because local climatic conditions may affect their efficacy if they are introduced (Abaajeh & Nchu 2015).

This study aims at investigating alternative methods for the control of the potato leaf miner. This was accomplished by investigating the potential of locally isolated EPN and EPF species as biological control agents against larvae and pupae of *L. huidobrensis*. Different life stages of the potato leaf miner were reared in the laboratory for screening under controlled laboratory conditions.

MATERIALS AND METHODS

Source of insects

Potato leaves infested with *L. huidobrensis* larvae were collected from different pivot irrigated fields from the farm Saamstaan, in the Sandveld, Western Cape province (32°35'19.3"S 18°20'07.9"E) (Fig. 4.1). The potato leaves were examined with the aid of a stereomicroscope, to determine mines with actively feeding larvae, which were cut out, avoiding damage to the epidermal layer of the leaf where the larva was feeding (Steyn *et al.* 2020). Pilot trials indicated high levels of mortality in the control treatment if the larvae were kept for more than 24 h. Hence, after field collection, the larvae were kept at ± 10 ºC before they could be counted and subsequently used in EPN and EPF screening trials. To obtain pupae, the larvae were kept until pupation at controlled temperatures of 25 ºC ± 2 ºC and 60 % relative humidity. After having reached the pupation stage, the pupae were stored at temperatures of ± 6 °C until use.

Fig. 4. 1. Map showing study sites from which the infected leaves were collected: The map on the top right is the South African map showing Sandveld region and the bottom left map is the magnified location of the fields.

Source of nematodes and fungi

Four locally isolated EPN species from South Africa were tested for pathogenicity against *L. huidobrensis* and two local EPFs were tested. Three EPN species, which include *Heterorhabditis bacteriophora* Poinar, *Heterorhabditis baujardi* Phan, Subbotin, Nguyen & Moens, and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams (Malan *et al.* 2006; Malan *et al*. 2011; Abate *et al.* 2017) were obtained from the collection of the Department of Conservation Ecology and Entomology, Stellenbosch University, whilst *H. zealandica* was the isolate [specifically from the potato survey (Chapter 3). The *M. robertsii* used in the experiment was isolated from the Sandveld whereas, the *B. bassiana* isolate was obtained from from the collection of the Department of Conservation Ecology and Entomology, Stellenbosch University.

Table 4.1. Different entomopathogens, with their associated host plants, origin and GenBank accession number, used in the study.

Species	Isolate	GenBank Accession number	Associated host	Origin
Heterorhabditis bacteriophora	SF351	FJ4558443	Grapevines	Wellington, Western Cape
H. baujardi	MT19	MF535520	Natural vegetation	KwaZulu-Natal
H. zealandica	S9	MW085080	Potatoes	Sandveld, Western Cape
Steinernema yirgalemense	157 C	EU625295	Citrus	Nelspruit, Mpumalanga
Beauveria bassiana	1ARC	MT355658	Apples	Grabouw (ARC), Western Cape
Metarhizium robertsii	14N	MW122513	Potatoes	Sandveld, Western Cape

The IJs of all nematode species (Table 1) were reared on *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and *Galleria mellonella* L. (Lepidoptera: Pylaridae) last instar-larvae at room temperature, and the harvesting and quantification procedures were according to the standard protocols described by Kaya & Stock (1997). Every day the IJs were harvested after they emerged from White traps for one week (Poinar *et al.* 1992). They were then stored horizontally in tissue culture flasks at 14 ºC according to procedures described by Kaya & Stock (1997) and used within one to three weeks of harvesting. On a fortnightly basis, the flasks were shaken to discourage nematodes from clumping, as well as to permit aeration of the suspension. The viability of nematodes was evaluated by inspecting the culture for mortality.

Bioassay screening protocol

The test arena included the use of 24-well bioassay trays (Flat bottom, Nunc™, Cat. No. 144530). However, only 10 wells were used in each of the trays and filled with filter paper discs (13 mm diam.). The filter papers were placed in alternate wells, to attain an even distribution throughout the bioassay plate. Each inoculation concentration was applied in 50 µl water for the different treatments, using an Eppendorf® micropipette. Insects (a single insect per well) were then added to the inoculated wells and the lid was, subsequently, closed. Five replicates of 24-well plates were used, with 10 wells in each, for each treatment tested (n = 50 insects). The 24-well bioassay plates were stored in 2-L plastic containers, and humidity was maintained by placing moistened tissue paper at the bottom of the container. Thereafter the container was placed in a growth chamber at 25 °C. After 48 h the insects were removed, and mortality was determined. In all cases mortality was confirmed by visual inspection of the insects, using a microscope.

Screening of nematodes against pupae

For the screening of *L. huidobrensis* pupae, the bioassay protocol as described above was followed. The pathogenicity of entomopathogens to *L. huidobrensis* was tested at the concentration of 200 IJs/50 µl distilled water per pupae. For the control treatment, 50 µl distilled water only was used. Each 24-well bioassay plate was covered using a sheet of glass, which was placed in the lid to prevent enclosed adults from escaping. The trial mortality was determined by squashing the pupae on a glass slide and then visually observing them for the presence of IJs. In all cases mortality was confirmed by visual infection with the nematodes or fungi. The experiment was repeated using a fresh batch of cultured IJs, leaf-mining larvae, and pupae on a subsequent date.

Screening of nematodes against larvae

Occupied mines with a single feeding larva was cut from the leaf and added to alternative wells of a 24-well bioassay plate according to the protocol described above. Larvae collected for the screening tests were of different ages, as it was not possible to determine the age or instar within their galleries (Steyn *et al.* 2020). The pathogenicity of EPNs to *L. huidobrensis* was tested at the concentration of 200 IJs/50 µl distilled water per larvae. Each well was closed and secured with a rubber band. The bioassay protocol as described above was followed and after 48 h the *L. huidobrensis* larvae were carefully removed from the leaf mines, and mortality was determined. In all cases mortality was confirmed by visual detection of infection with the nematodes. The experiment was repeated using a fresh batch of cultured IJs and leaf-mining larvae on a subsequent date.

Lethal nematode dosage

The determination of the lethal dosage was done by using the most virulent EPN species from the previous experiment. Three EPN species doses 200, 100, and 50 IJs per larvae/pupae were used to determine lethal concentration levels. Again, the 24-well bioassay tray protocol was used in this experiment. A single *L. huidobrensis* larva or pupa was placed in each well. The control was untreated; only 50 µl of distilled water was added. Insect mortality was checked after 48 h, and the presence of nematodes inside dead larvae/pupae was observed to confirm nematode mortality by infection for each insect stage.

Screening of fungi against pupae

Preparation of conidia inoculum

The fungal conidia used in these experiments were harvested from the culture surfaces by scrapping them from the (Saboraud dextrose with yeast) plates (Coombes 2012). Conidia was collected and suspended in autoclaved distilled water of 20 ml with 0.05 % Tween 20, which were placed in 28 ml McCartney wide-mouth glass bottles. These bottles were later sealed and vortex-mixed for 2-3 min until a homogenous suspension was obtained, then poured through organza fabric into a sterilized 100 ml glass beaker, to remove the pieces of agar and mycelium present, poured into the 28 ml McCartney bottles and vortex-mixed for 2 min. This concentrate became the conidial stock for the serial dilutions concerned.

A Neubauer haemocytometer was used to determine the conidial concentration and adjusted to provide 1×10^7 conidia per ml^{-1} suspensions of each isolate for the laboratory experiment. Then the number of spores were counted using a compound microscope on both ends of the haemocytometer. After the above procedure, a specific formula to determine conidial concentrations was used: The total number of spores counted on each side of the haemocytometer was added, and the average was established, which was then multiplied by 5×10^4, to give a concentration of 1×10^7 conidia/ml (Inglis *et al.* 2012). However, to calculate the exact volume of the conidial suspension and that of distilled water, the following formula was used: $C_1V_1 = C_2V_2$. Where: C_1 = initial concentration; V_1 = required volume of conidial suspension stock to be added to the distilled water; C_2 = desired concentration, and V_2 = desired final amount of inoculum.

Screening of pupae

The protocol described for the screening of pupae above was followed and the 50 pupae per treatment were inoculated with 1×10^7 conidia per pupae *B. bassiana* and *M. robertsii*. The mortality of the potato leaf miner was recorded five days post-treatment with the four different fungal isolates. Insect mortality was confirmed through growing the insect cadavers on SDAY plates and incubating them at 25 °C ± 2 °C (Coombes 2012). After seven days, following mycosis on the insect body and the fungal growth on the SDAY plate, the plates were checked, and the dead insects evaluated with the aid of a microscope.

Statistical analyses

Analysis of the results was performed using STATISTICA 13.3 (TIBCO Soft Inc. 2016). The data were analysed using a one-way ANOVA (Analysis of variance). The mean lethal values (LD_{50}, LD_{90} and LD_{95}) were calculated using probit analysis at 95 % confidence intervals.

RESULTS

Screening of nematodes against pupae

The analysis of the screening data showed that all the EPN species caused significantly higher mortality of *L. huidobrensis* pupae than the untreated control treatment ($F_{4, 70} = 23.43$ $p < 0.01$). Mortality associated with *H. baujardi* (57.3 % ± 3.65 %) and *H. zealandica* (49.3 % ± 3.65 %) did not significantly differ ($p = 0.13$) from each other and obtained the highest mortality in the screening trials. The natural mortality of the control treatment was 11.5 % ± 3.65 % (Fig. 4.2). Virulence of *H. bacteriophora* (44.8 % ± 3.65 %) did not differ significantly from *H. baujardi* ($p = 0.02$) and *S. yirgalemense* (46.5 % ± 3.65 %). However, the virulence associated with *H. baujardi* differed significantly ($p = 0.04$) to that of *S. yirgalemense*.

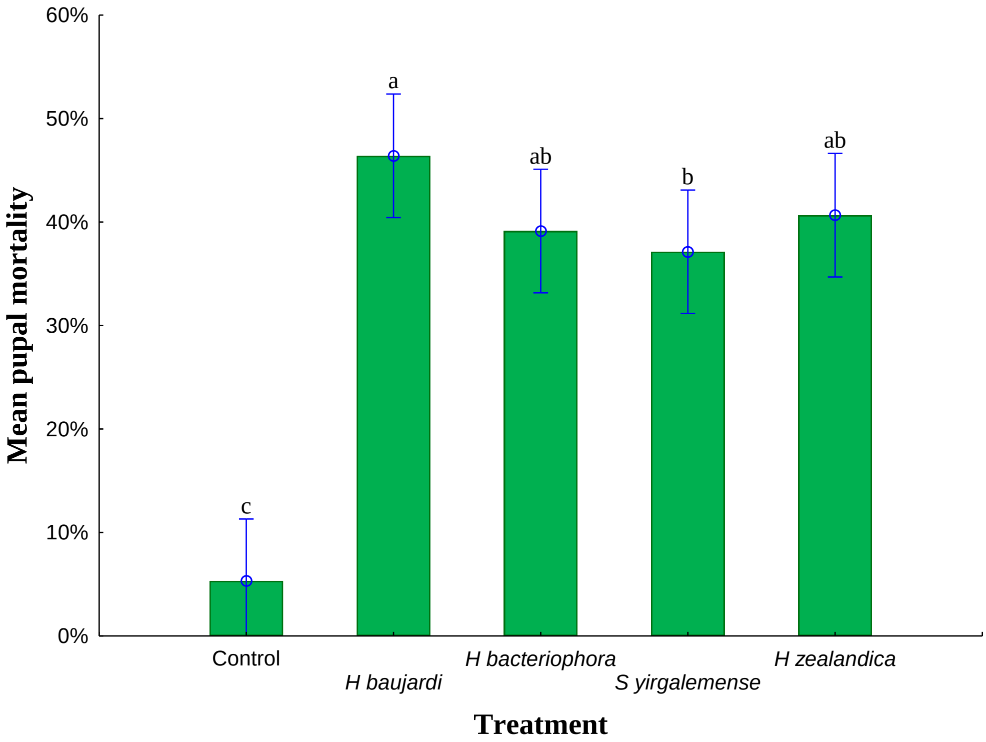

Fig. 4. 2. The percentage mortality of *Liriomyza huidobrensis* pupae (95 % confidence intervals) caused by the three *Heterorhabditis* and one *Steinernema* species tested using a concentration of 200 IJs/50 µl distilled water and distilled water only control. Devaitions in lettering above the bars indicate significant differences between treatments ($P < 0.05$) and the mortality of the pupae.

Screening of nematodes against larvae

The highest percentage mortality of *L. huidobrensis* larvae was obtained with *H. baujardi* (71.7 % ± 4.08 %), showing significant differences ($p < 0.01$) between different species. *Heterorhabditis bacteriophora* (51.5 % ± 7.67 %) was also significantly different from the rest of the treatments ($p < 0.01$). On the other hand, the percentage mortality of *S. yirgalemense* (64.4 %

± 6.24 %) and *H. zealandica* (56.05 % ± 3.80 %) did not significantly differ ($p > 0.12$) from each other. The analysis of the screening data showed that all the screened EPN species caused significantly higher mortality of *L. huidobrensis* larvae than the untreated control treatment ($F_{4, 20} = 120.61$; $p < 0.01$) (Fig. 4.3).

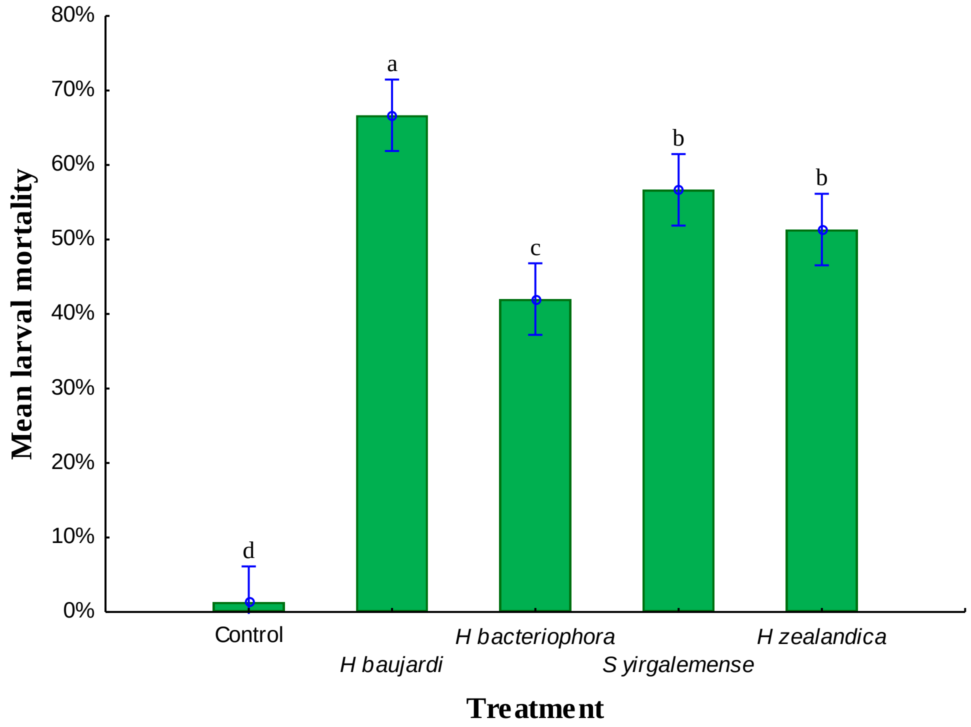

Fig. 4. 3. The percentage mortality of *Liriomyza huidobrensis* larvae (95 % confidence intervals) caused by the four entomopathogenic nematode species tested, using a concentration of 100 IJs/50 µl distilled water and a water only control. Deviations in lettering above the bars indicate significant differences between treatments ($p < 0.05$) the nematode treatments and the mortality of the larvae**.**

Screening of fungi against pupae

Significant differences were found in pupal mortality (infection) caused by the different EPF species. The two screened EPF caused significantly higher mortality of *L. huidobrensis* pupae than the untreated control treatment ($F_{2, 12} = 165.47$; $p < 0.01$), after 48 h. Significantly higher mortality (infection) was associated with *M. robertsii* (81.4 % ± 3.12 %), compared to *B. bassiana* (67.6 % ± 3.12 %), which were significantly different from each other ($p < 0.01$) (Fig. 4.4).

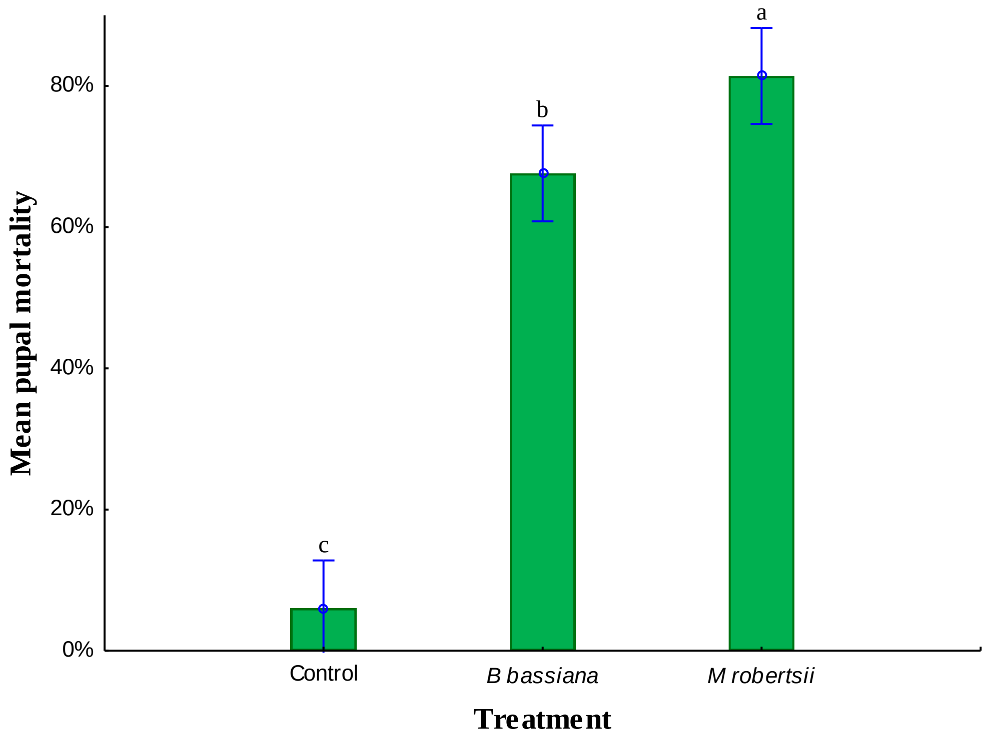

Fig. 4. 4. The percentage mortality of *Liriomyza huidobrensis* pupae (95 % confidence intervals) caused by the two entomopathogenic fungi species tested, at a concentration of 1×10^{7} conidia per ml^{-1} and distilled water and 0.05% Tween 20 as control. Deviations in lettering above the bars indicate significant differences ($p < 0.05$) in pupal mortality caused by the respective fungal treatments.

Lethal dosage of entomopathogenic nematodes against larvae and pupae

The lethal dose trials against *L. huidobrensis* larvae exposed to four different nematode concentrations showed a positive relationship as there was an increase in the total percentage mortality with an increase in dosage (Fig. 4.5). Variability in virulence levels was obtained with the different concentrations. Generally, the insect infection with the EPNs was low, with all the EPN species obtaining infection of larvae below 40 % at the concentration of 50 IJs/larva. There was an increase in infection at 200 IJs for all the EPN species tested, with effectiveness ranging between 43 % - 68 %.

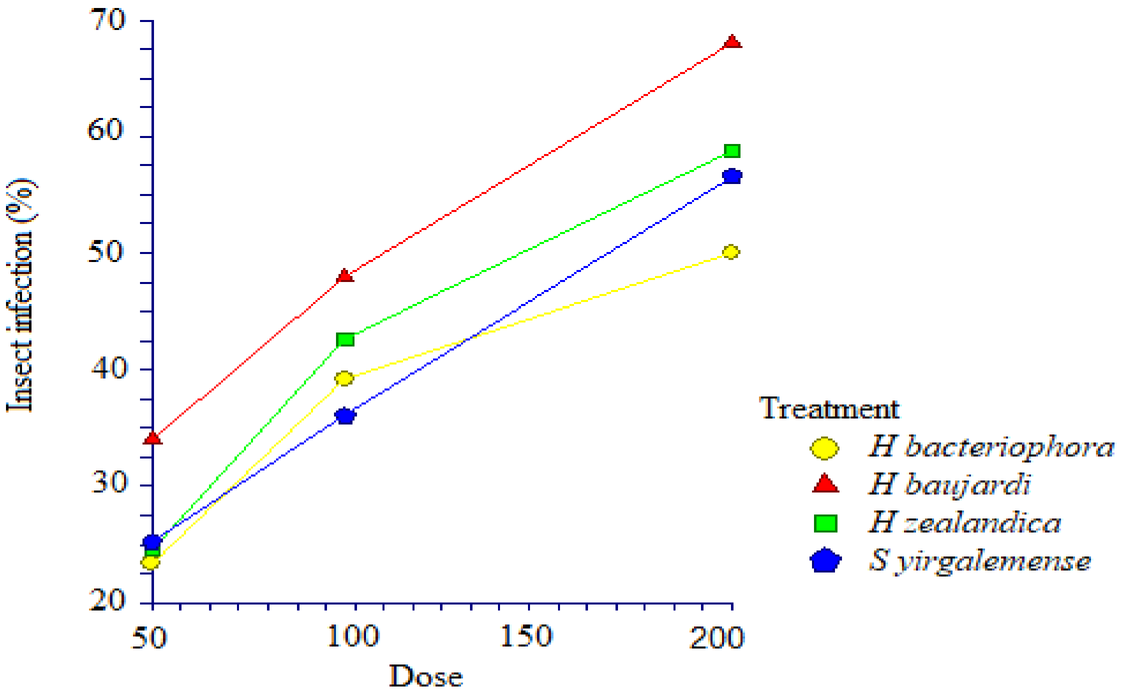

Fig. 4. 5. Average percentage mortality of *Liriomyza huidobrensis* larvae, following exposure to four different IJ concentrations (50, 100 and 200 IJs per 50 µl distilled water) of *Heterorhabditis bacteriophora, H. baujardi, H. zealandica* and *Steinernema yirgalemense*.

The Probit analysis on larvae (Fig. 4.6) indicated the data fits the probit model with the following impatiatiens estimations *Heterorhabditis baujardi* (Chi-square = 1.25; DF = 1; Probability level = 0.26), *H. bacteriophora* (Chi-square = 1.59; DF = 1; Probability level = 0.21), *H. zealandica* (Chi-square = 0.43; DF =1; Probability level = 0.51), and *S. yirgalemense* (Chi-square = 2.30; DF = 1; Probability level = 0.13).

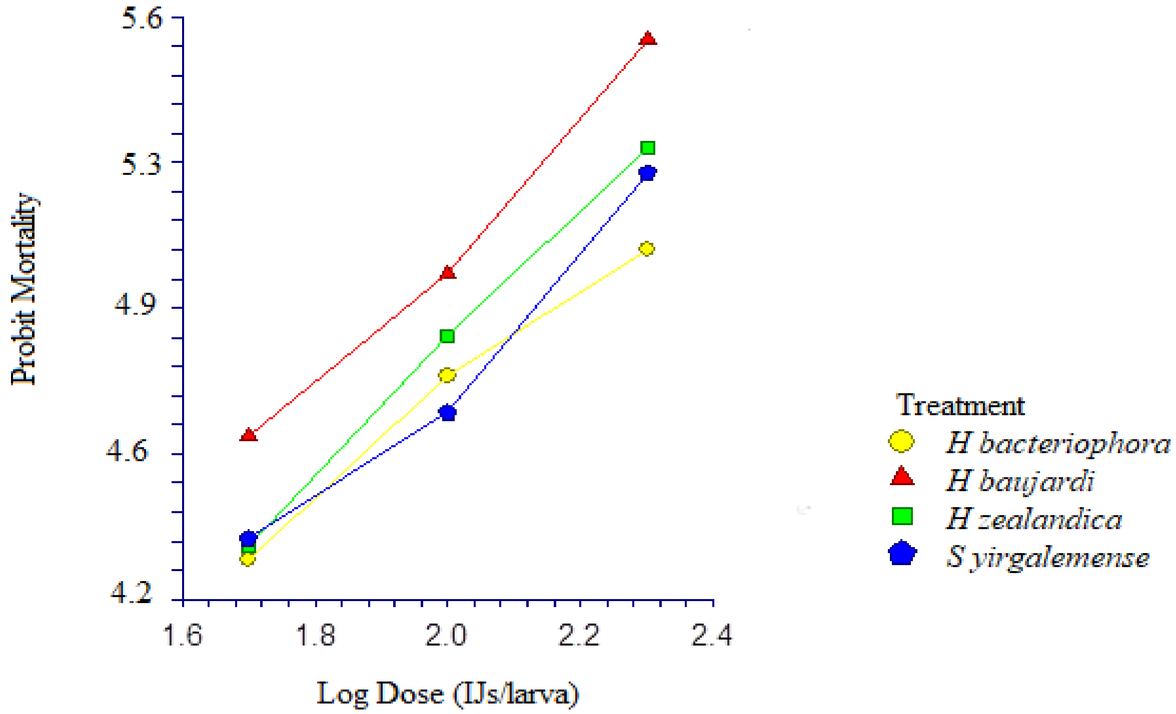

Fig. 4. 6. Probit mortality of larvae obtained at each log concentration tested for *Heterorhabditis bacteriophora*, *H. baujardi*, *H. zealandica* and *Steinernema yirgalemense* against *Liriomyza huidobrensis* larvae after 48 h.

The exposure of pupae to different doses of EPNs showed a positive relationship. However, low mortalities were observed with all the tested treatments at all concentrations. *Heterorhabdtis baujardi* caused the highest mortality of 30 %, 48 % and 61.2 %, respectively with 50 IJs, 100 IJs and 200 IJs. Most of the treatments did not reach 50 % effectiveness at 200 IJs/pupa. There was an increase in mortality with an increase in dose. However, that was not the case for *S. yirgalemense*, which showed a slight reduction in mortality from 46 % at 100 IJs/pupa to 43.4 % at 200 IJs/pupa (Fig. 4.7).

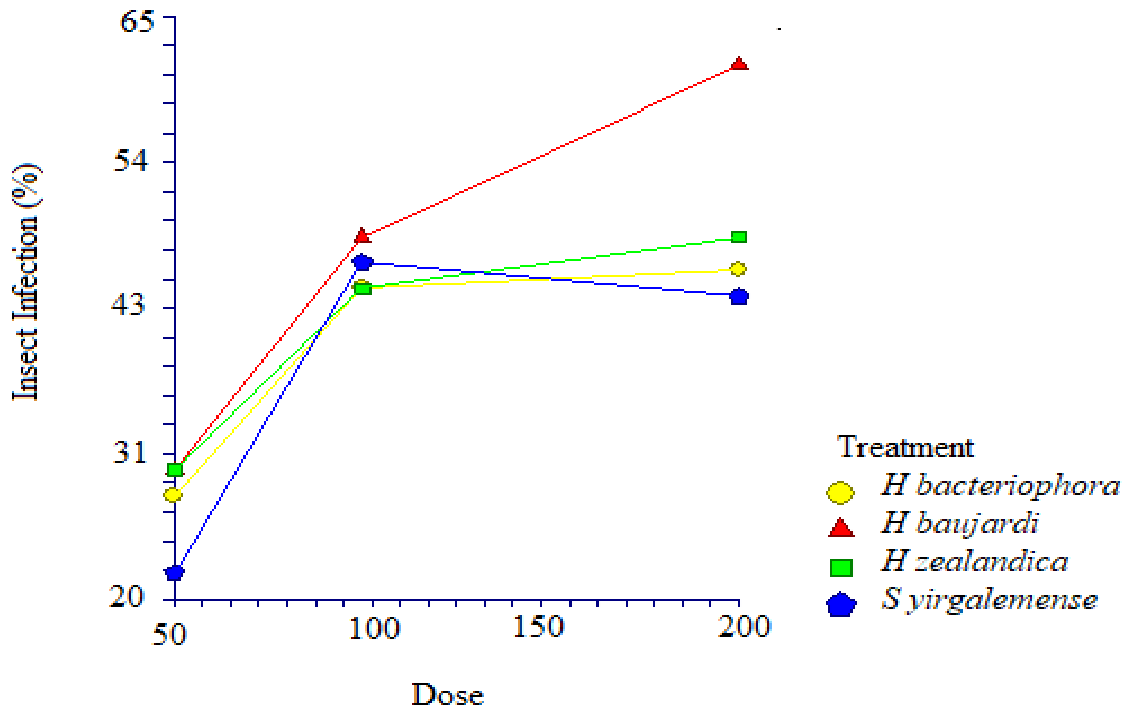

Fig. 4. 7. The effect of dose [number of infective juveniles (IJs) per pupa] of four entomopathogenic nematode species (*Heterorhabditis bacteriophora*, *H. baujardi*, *H. zealandica* and *Steinernema yirgalemense*) on the infection of *Liriomyza huidobrensis* larvae at concentrations of 50, 100 and 200 IJs per 50 µl distilled water.

The Probit analysis (Fig. 4.8) indicated the data fits the probit model with the following impatiatiens estimations; *H baujardi* (Chi-square = 1.01; DF = 1; Prob level = 0.31), *H. bacteriophora* (Chi-square = 8.15; DF = 1; Probability level = 0.00), *H. zealandica* (Chi-square = 3.86; DF = 1; Probability level = 0.05), and *S. yirgalemense* (Chi-square = 27.93; DF = 1; Probability level = 0.00).

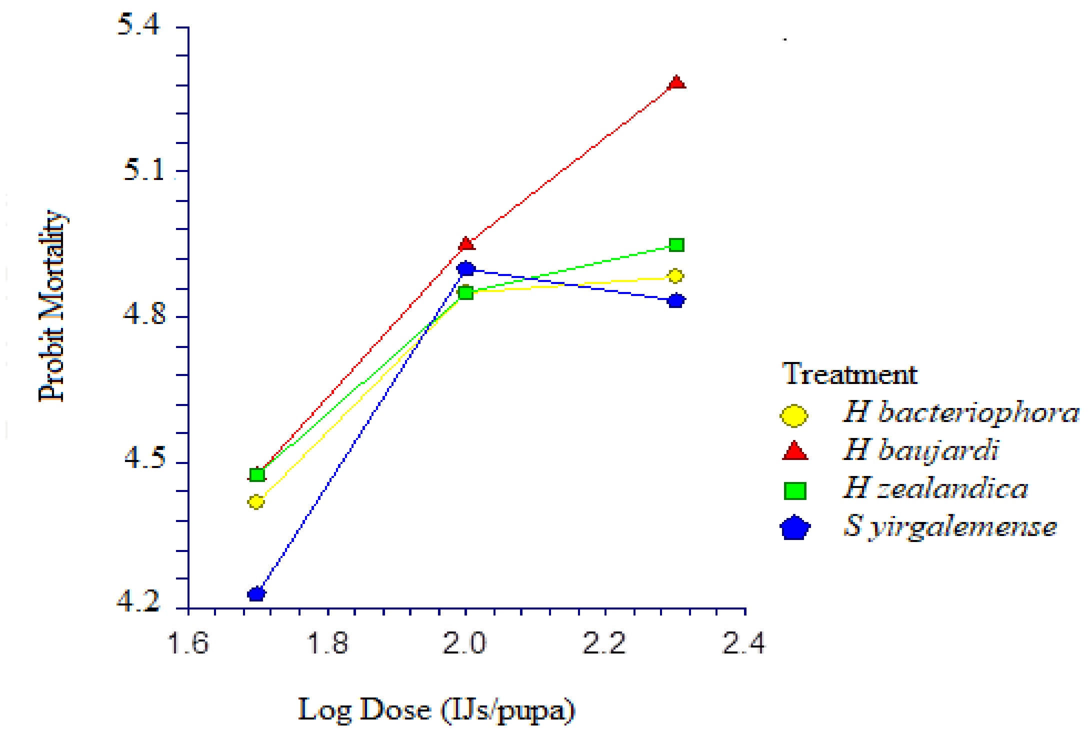

Fig. 4. 8. Probit mortality of pupae obtained at each log concentration tested for *Heterorhabditis bacteriophora, H. baujardi, H. zealandica* and *Steinernema yirgalemense* against *Liriomyza huidobrensis* larvae after 48 h.

The treatments that exhibited the lowest LD_{50}, LD_{90} and LD_{95} in the pupae trials was *H. baujardi* with values of 233.38, 11130.15 and 33290.17, respectively, whereas *H. bacteriophora* had the highest LD_{50}, LD_{90} and LD_{95} with values of 233.38, 11130.15 and 33290.17, respectively (Table 4.2). On the other hand, with regards to the larvae trials the treatment with the lowest LD_{50}, LD_{90} and LD_{95} again was *H. baujardi* indicating values of 99.93,752.73, and 1334.27 respectively, whilst *H. bacteriophora* again had the highest LD_{50}, LD_{90} and LD_{95} with values of 189.489, 2235.13, and 4499.04, respectively (Table 4.3).

Table 4. 2. The lethal dose (LD) of infective juveniles against *Liriomyza huidobrensis* pupae inoculated with varying concentrations of *Heterorhabditis baujardi*, *H. bacteriophora*, *H. zealandica* and *S. yirgalemense*.

	H. bacteriophora		***H. baujardi***		***H. zealandica***		***S. yirgalemense***	
Lethal dose	Dose	Std. error Dose	Dose	Std. error Dose	Dose	Std. error Dose	Dose	Std. error Dose
LD_{50}	233.38	41.23	117.86	6.93	203.44	31.26	224.10	30.32
LD_{90}	11130.15	9289.86	1064.31	260.75	8923.83	6928.95	4754.55	2602.10
LD_{95}	33290.17	34192.25	1986.10	609.40	26065.62	25020.79	11303.22	7558.42

Table 4. 3. The lethal dose (LD) of infective juveniles against *Liriomyza huidobrensis* larvae inoculated with varying concentrations of *Heterorhabditis baujardi*, *H. bacteriophora*, *H. zealandica* and *Steinernema yirgalemense.*

	H. bacteriophora		***H. baujardi***		***H. zealandica***		***S. yirgalemense***	
Lethal dose	Dose	Std. error Dose	Dose	Std. error Dose	Dose	Std. error Dose	Dose	Std. error Dose
LD_{50}	189.49	17.90	99.93	5.21	139.16	8.06	160.83	11.27
LD_{90}	2235.13	802.32	752.73	147.16	986.56	209.99	1331.20	344.06
LD_{95}	4499.04	1972.52	1334.27	330.10	1718.93	451.20	2425.20	767.63

DISCUSSION

In nature, EPF and EPN are key components in the regulation of insect populations (Mei *at al*. 2020; Labaude & Griffin 2018). The process of regulating insect populations by EPNs is by using insect hosts as a food source, basically reproducing and breeding within the insect host (Griffin *et al.* 2005). Therefore, in this study we examined the pathogenic potential of two EPF species and four EPN species against the larvae and pupae stages of *L. huidobrensis.* The results of the present study demonstrated the ability of *B. bassiana* and *M. robertsii* to induce infection and ultimately cause mortality of *L. huidobrensis* even though the effectiveness varied depending on the conidia concentration of the fungi. All the local EPF species tested caused a significant increase in pupae mortality of *L. huidobrensis* compared to the control (untreated) treatments, signifying their ability for spores to attach and infect, with mortality with *M. robertsii* (81.4 % ± 3.12 %) and *B. bassiana* (67.6 % ± 3.12 %) treatments, respectively, and overt mycosis being observed on the seventh day for pupae inoculated with *B. bassiana* and *M. robertsii*, whereas the control treatment indicated no sporulation. In a similar study by Noujeim *et al*. (2015), *B. bassiana* caused *L. huidobrensis* pupae mortality ranging between 73 % and 93 % depending on the concentration and sporulation between 13 % and 50 % were recorded on pupae seven days after the treatments were applied. Thus, the ability for fungi to infect *Liriomyza* pupae and ultimately cause mortality has been demonstrated (Noujeim *et al*. 2015; Gathage *at al.* 2016). All the local EPNs tested caused a significant increase in larval and pupal mortality of *L. huidobrensis* compared to the control treatments, this signifies the ability for them to gain access and infect the target insect pest through natural openings. The EPN species that caused the highest mortality at the pupal stage was *H. baujardi* even though the mortality was generally low with mortalities ranging between 35.6 % and 55.2 %. The EPN species tested caused between 30 % and 51 % mean pupal mortality. Virulence differed significantly between the control and the EPN species tested, however, there were no significant differences observed between most treatments. However, only *H. baujardi* was significantly different from *S. yirgalemense.* The puparium of *L. huidobrensis* is oval and has a slight flat surface ventrally, it is generally small measuring between ca. 1.3 to 2 mm in length and ca. 0.5 mm wide, thus the lower percentage mortality observed could be attributed to the size of the EPN species used. Bastidas *et al*. (2014) identified that the size of the nematode species plays a vital role in its effectiveness in regulating insect populations. Several studies have confirmed that the size of nematodes does matter, especially when working with micro-bodied hosts. The study by Katumanyane *et al.* (2018) showed that the larger EPN species, such as *Steinernema khoisanae* Nguyen, Malan & Gozel, (Rhabditida: Steinernematidae), had very low control potential against the devastating *Bradysia impatiens* Johannsen (Diptera: Sciaridae), a small fly found on undercover crops and greenhouses, which clearly resonates with the low susceptibility potential obtained from *Steinernema yirgalemense*, with low mortality in all the trials.

The efficacy of EPNs against larvae varied with the species. The susceptibility of *L. huidobrensis* larvae against the entomopathogenic nematode *H. baujardi* was relatively high in our experiments compared to the other species (71.7 % ± 4.08 %), which could be attributed to its size. However, *H. zealandica* and *H. bacteriophora*, despite having smaller body sizes, showed lower mortalities as compared to *S. yirgalemense* with a larger body size (Van Damme *et al*. 2016), which obtained the highest mortality with *Steinernema feltiae* Filipjev and *Steinernema carpocapsae* Weiser, whilst *H. bacteriophora* showed lower mortalities when evaluated against the *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) larvae. This variation could be as result of the differences in pathogenicity of the symbiotic bacteria associated with different EPN species, as reported by (Yooyangket *et al*. 2018).

Liriomyza huidobrensis can easily develop resistance to chemicals and as such biological control using EPNs offers alternative approaches to reducing its spread. The study indicated a definite positive relationship between the concentration of all the four nematode species used for inoculation and the percentage mortality of *L. huidobrensis*. In a similar study, Steyn *et al*. (2019) also obtained a positive relation between mortality and concentration when different EPNs were tested for pathogenicity against *Holocacista capensis* Van Nieukerken & Geertsema (Lepidoptera: Heliozelidae). According to Wright *et al.* (2005), maximizing the density and distribution of EPNs on the leaf surfaces may increase the ability of the nematode species to locate an entrance to the mine of a leaf-mining insect. With regard to the dose necessary to kill more than 50 % of both larval and pupal life stages of *L. huidobrensis*, *H. baujardi* had the lowest LD_{50} while *H. bacteriophora* and *S. yirgalemense* had the higher LD_{50}, which could be attributed to both the size of the nematode species and the pathogenicity potential of the symbiotic bacteria (Wright *et al.* 2005; Steyn *et al*, 2019). In the current study, LD_{50} values for *H. baujardi* larvae (99.93) were the lowest. However, the values obtained were higher than those obtained by Steyn *et al.* (2019) (LD_{50} = 9.69) who evaluated different entomopathogenic nematodes against *H. capensis* (Cape grapevine leaf miner). This could be associated with the ability of nematodes to penetrate the mines, find larvae, and cause mortality, which has been reported in another leaf mining insect, *Tuta absoluta*, a pest of tomatoes (Batalla-Carrera *et al*. 2010; Van Damme *et al*. 2016). In conclusion, the findings of this study indicate that locally sourced EPNs and EPF have the potential to control *L. huidobrensis* pupae and larvae and as such are considered potential biocontrol agents for the invasive leaf miner. These results, therefore, provide useful information on the ability of EPNs to penetrate pupae and larvae (mines) and the possibility of controlling pupae and larvae using fungal pathogens and entomopathogenic nematodes. Adopting such biocontrol agents will helpreduce the need of chemical insecticides.

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CHAPTER 5

GENERAL DISCUSSION

Liriomyza huidobrensis Blanchard (Diptera: Agromyzidae), or the potato leaf miner, is an economically important pest of potato crops around the world and South Africa is no exception. To date, there is evidence regarding the temporal variations in population dynamics between different cultivars. The potato leaf miner affects potatoes at all phenological stages and causes damage at tuber maturation. Therefore, for effective control, chemical insecticides have been widely used. Biological control agents such as entomopathogenic fungi and nematodes have been successfully used for the control of many insect pests worldwide.

Liriomyza huidobrensis is an important pest in potato production. However, there is little to no information regarding its population dynamics, and biological control options in South Africa. Therefore, the overall aim of this study was to study the bio-ecology and determine biological control options for the leaf miner on potatoes in the Western Cape province specifically, by understanding the dynamics of leaf miner flies both within the actual field and surrounding natural vegetation. Thus, the first objective of this study involved determining the mode of infestation of *L. huidobrensis* through population dynamics and determination of associated parasitoids. The above was undertaken by means of placing sticky traps within the potato fields and in the srrounding natural vegetation at predetermined distances. The traps were then monitored until tuber maturation. The results of this study showed a clear association between crop development and *L. huidobrensis* population densities, which increased steadily from as early as week 3 after plant emergence to tuber maturation. The population of the adult potato leaf miner in winter was slightly lower than that of the summer population. On the other hand, regarding leaf infestation levels, the highest leaf damage was observed on the lower leaves when compared to the upper leaves. López *et al.* (2010) found similar trends and concluded that it may be related to the extrusion phenomenon, which comes with leaf aging in that extrusion is low in lower/older leaves and high in upper/new leaves. The concept of egg extrusion exposes the egg to the environment and consequently to the natural enemies through hypertrophic cell growth, which causes egg ejection (López *et al.* 2010).

On average the leaf infestation levels reached 50 % in week 7 and the highest adult population densities were obtained between week 6 and 10 after plant emergence. The survey also demonstrated that *L. huidobrensis* is well established in both cultivated potato plants and weeds. However, few samples of other *Liriomyza* species were identified in the Western Cape province. Some samples of *Liriomyza sativae* were found in the Sandveld area, which was confirmed through molecular identification. In the laboratory, colony establishment proved to be useful using bean plants for larval development, due to their large leaf surface area, which permitted more punctures on one single leaf. The other advantage was that bean plants were fast growing compared to tomato plants.

During the study, a low diversity in parasitoids (*Diglyphus isaea*, *Alysiinae* sp., *Utetes africanus*, *Eulophinae* sp., and *Dacnusa sibrica*) was observed compared to the high diversity in *Liriomyza* parasitoids found along the Peruvian coast where more than 17 000 parasitoid species were discovered (Mujica & Kroschel 2011). Differences in altitudes, host plants and temperatures contributed to the differences in parasitoid abundance. *Diglyphus* sp. has been the common parasitoid species recorded in South Africa (Weintraub *et al*. 2017) and was also the most abundant species obtained in our study. This study is the first to document parasitoids of the potato leaf miner in the Western Cape province. The lack of diversity in parasitoids associated with *L. huidobrensis* could be attributed to the constant application of chemicals in these fields, which are applied every two weeks, because studies suggest that chemical insecticides contribute significantly to natural enemy reduction (Shepard & Braun 1998; Salvo & Valladares 2007) as well as factors like altitude, host presence and temperature, which play a vital factor in parasitoid distribution (Hance 2007; Tantowiyo & Hoffman 2010) and perhaps the parasitoids were just low in numbers during the time of the survey. In South Africa, BioBee has developed a product called Bio® Diglyphus, which is the parasitic wasp *Diglyphus isaea* for leaf miners. Also, augmentative release of *D. isaea* and *Dacnusa sibirica* were recorded in Egypt in as early as 2006 on vegetable crops in greenhouses (Abd-Robou 2006) and considered the most abundant parasitoid species in Israel (Weintraub and Horowitz 1995).

The second objective of the study involved a survey of EPNs (entomopathogenic nematodes) and EPF (entomopathogenic fungi) found in local potato fields, which was undertaken by means of collecting soil samples. The collected soil samples were baited for the presence of EPF and EPNs using susceptible host insects. The collected EPF isolates and EPN isolates were identified, both morphologically and molecularly, to species level. From the survey of 16 fields, two EPF species were successfully isolated, namely *Metarhizium robertsii* and *Metarhizium majus* and different isolates of *Heterorhabditis zealandica* (Chapter 3). All the collected EPF and EPN isolates were found in potato fields of the Sandveld. The results indicated little diversity of EPF and EPN isolates in the potato fields of the Sandveld, Western Cape, with less than 40 % of the 16 surveyed fields showing entomopathogens. Furthermore, the different *M. robertsii* isolates and *M. majus* were screened for pathogenicity potential against the pupae stage of *L. huidobrensis*, the results of which showed that different strains of the same species have different pathogenicities. This could possibly be associated with genetic variation between pathogen. A similar study by Tiago *et al.* (2011), found differences in pathogenicity of *M. anisopliae* against the larval stage of the sugarcane root spittlebug *Mahanarva fimbriolata* (Stål) (Hemiptera: Cercopidae).

The final objective was to determine the pathogenicity of the entomopathogenic fungi (*Beauveria bassiana* and *M. robertsii)* and nematode isolates from the potato field (*H. zealandica*) and some other locally isolated entomopathogenic nematodes (*H. baujardi, H. bacteriophora* and *S. yirgalemense*) to different life stages of *L. huidobrensis.* The EPF species were tested against the pupae stage of the leaf miner, but insect infection varied depending on conidia concentration and the fungal isolates. The highest performing fungal isolate against the pupae stage was *M. anisopliae* with more that 80 % mortality recorded, while *Beauveria bassiana* performed a bit lower (68 %) but generally well. All the tested EPNs caused significant mortality of pupae and larvae at different concentrations. The nematode species *H. baujardi* caused higher mortality of *L. huidobrensis* because of its smaller size, compared to the larger sized nematodes, such as *S. yirgalemense.* This study therefore provided information on the possibility of using EPNS and EPFs as biocontrol agents of different life stages of the potato leaf miner.

This study provides insight on the alternative control measures and benefits of integrating locally isolated EPF and EPNs in the management of the potato leaf miner, which is an important pest of potato plants (Weintraub *et al.* 2017). Based on the results of the present study, EPNs and EPF can cause mortality to the larvae and pupae stage of *L. huidobrensis,* therefore, the potential of field applications should be investigated. In the present study, the larval stage was the most susceptible to EPNs, therefore, foliar application is widely encouraged (Tomalak *et al.* 2005), using optimal EPN concentrations and suitable application techniques that enable proper distribution of EPNs on the leaf surface. The adoption of biological control measures like EPNs for controlling *L. huidobrensis* pupae would minimise leaf miner populations in potato fields and the use of chemical insecticides (CABI 2019). Furthermore, application of nematodes on the soil against soil-inhabiting pupae before potato planting, could reduce the number of adults that will hatch from the soil. The higher temperatures during the summer monitoring period might have favoured the development of *L. huidobrensis* populations and contributed to the high mean trap catches obtained during this period. Similarly, Rodríguez-Castañeda *et al.* (2017) reported a higher percentage of adult emergence of *L. huidobrensis* and *Liriomyza sativae* when temperatures where high as compared to when they were low during a study in Guatemala. The application of nematodes for the summer growing season should, therefore, be done before planting. Low temperatures have been shown to prolong development of *L. huidobrensis* from the egg to adult stage whereas high temperatures shorten the development period (Mujica *et al.* 2017). There are so many biotic and abiotic factors that affect the efficacy of nematodes during field applications, including target insects, life stage and temperature. Temperature play a vital role in the effectivity of EPNs under field conditions as higher temperatures are directly associated with nematode desiccation and reduce nematode survival (Shapiro-Ilan *et al.* 2006). However, nematodes can provide efficancy in field applications for a period of two to eight weeks after application under optimal conditions (Shapiro-Ilan *et al.* 2012). Given the fact that the summer growing season begins in October in South Africa, an early soil application of EPNs before potato planting, to control the pupae stage of the leaf miners that may be overwintering, could be considered as temperatures of 25 °C are favourable and will not promote desiccation of nematodes. During the monitoring period the highest population densities were observed between August and September for the winter monitoring period, whereas December and January had the highest catches for the summer monitoring period in the present study and as such a winter application of nematodes should be done before planting and at tuber initiation, which is usually around week 6 and 8 after plant emergence. Applications during the summer period should be accompanied with constant irrigation for maintenance of adequate moisture conditions (Shapiro-Ilan *et al.* 2012). Proper timing in the application of different chemical and biological insecticides is a vital component in the implementation of an effective integrated management program, as any treatment that is wrongly timed leads to poor insecticide performance and ultimately increases production costs (Ekesi *et al*. 2001). Thus, fungi applications under field conditions should involve morning applications of the fungi to avoid the conidia from being blown away by the wind and drying out.

Reservoir weed plants play a vital role in the spread of invasive agricultural pests, especially when the pest attacks a wide range of plants and has high reproductive fitness (Abbes *et al*. 2016). Thus, the weeds could be potential threats by harbouring leaf miners. *Liriomyza huidobrensis* is a highly polyphagous pest and has no clear preference to any plant family (CABI 2019). Therefore, the common weeds found in the natural vegetation, including daisies *Arctotis breviscapa* Thunb., Saldanha Pincushion (*Leucospermum tomentosum*), graafwater shrubs, *Brachylaena discolor* and *Helichrysum* may have contributed to the few leaf miners found during the off season monitoring when no potatoes where planted and may contribute to leaf miner presence, although numbers were extremely low. In conclusion, the findings of this study will contribute to the information gap that exists regarding the bioecology and ecology of the potato leaf miner in the Western Cape province of South Africa. The identification and screening of local EPNs and EPF showed potential to control *L. huidobrensis* pupae and larvae, due the high pathogenicity obtained, which motivates follow up studies to determine how long both nematodes and fungi persist in the soil, determine the mode and optimum time of application of EPNs and EPF. Adopting such biocontrol agents will help in the reduction of chemical insecticide overuse.

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