Investigating biological control agents for the management of *Ceratitis capitata* (Wiedemann)

by

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Declaration

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Abstract

Persistent fruit damage and loss caused by fruit flies (Diptera: Tephritidae) has occasioned the reliance on chemical control methods for their management in the fruit industry. However, social, environmental and economic consequences associated with such control methods have necessitated the need for the exploration of alternative, more sustainable and eco-friendly options. This study investigates the use of entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPF) and parasitoid wasps, as biological control agents against one of the most widespread and dominant fruit flies in South Africa, the Mediterranean fruit fly or Medfly (*Ceratitis capitata*). Different methods were used in order to (i) isolate and identify local EPNs and EPF from fruit orchard soils; (ii) evaluate the pathogenicity of local EPN and EPF isolates against the third larval instar stage of Medfly under controlled laboratory conditions, and selected species of each in a more natural (sand) environment; (iii) estimate the lethal concentration/dose needed to result in 50% *C. capitata* mortality (LD_{50}) using selected EPN isolates; and (iv) survey for and identify fruit fly parasitoid species occurring in the Western Cape, Mpumalanga and Limpopo Provinces. Soil sampling yielded a number of local entomopathogenic isolates, including EPNs with new bacterial associations. Similarly, an EPF, *Metarhizium robertsii* (MJ06), was also trapped using Medfly larvae as bait. Initial EPN screenings (100 IJs / 50 µl) showed all tested EPNs to be highly pathogenic against third instar Medfly larvae, while at the lower concentration (50 IJs / 50 µl), *Heterorhabditis noenieputensis*, was the most virulent EPN species. This species, as well as *Steinernema yirgalemense*, which is currently in the process of being mass cultured and formulated for commercial use, was further, tested in sand bioassays. *H. noenieputensis* caused significantly higher mortality (94-100%) as most Medfly infected as larvae pupated, but died within the puparium. *S. yirgalemense* also offered good control, with 58-79% of exposed larvae dying as adults. The LD_{50} of *H. noenieputensis* was 37 IJs / insect, which was 14 times more effective than that of *S. yirgalemense.* Local EPF isolates and commercial products tested against third instar larvae, using the dipping method, were pathogenic and caused visible fungal infection (mycosis) of 57-74%. Reduction of humidity also reduced overall mycosis, with the highest mycosis of 55% due to the local isolate, MJ06. Third instar Medfly larvae added to sand and sprayed with the soil-collected EPF *M. robertsii* (MJ06) and *Beauveria bassiana* (6756), died and mycosed as adults (62-86%). Parasitoid wasps were obtained during fruit sampling, but difficulties with low DNA extraction, amplification and limited available barcodes of local fruit fly parasitoids, restricted their species identification. The use of sentinel traps - setting out apples infested with Medfly eggs, larvae and exposed pupae - did not trap any wasps during this study, but provides a simple and inexpensive method to be used in future studies. This study documents an EPN (*H. noenieputensis* SF669) and EPF (*M. robertsii* MJ06), virulent against the soil-life stages of Medfly, which could be the focus of future studies as potential biocontrol agents. Moreover, this study provides novel data on additional biological control agents that could be incorporated into an overall integrated pest management system (IPM) system, to sustainably and effectively manage the Mediterranean fruit fly

Opsomming

Die voortdurende skade en verliese veroorsaak deur vrugtevlieë (Diptera: Tephritidae), het die vrugtebedryf forseer om afhanklik te wees van chemiese beheermetodes. Negatiewe sosiale, omgewings en ekonomiese gevolge wat met hierdie metodes gekoppel is, het die soektog na alternatiewe, vir meer volhoubare en omgewingsvriendelike, beheermiddels genoodsaak. Hierdie studie ondersoek die gebruik van entomopatogeniese nematodes (EPNs), entomopatogeniese swamme (EPF) en parasitiese wespe, as biologiese beheeragente vir die wydverspreide vrugtevlieg in Suid-Afrika, die Mediterreense vrugtevlieg of Medvlieg (*Ceratitis capitata*). Verskeie metodes was gebruik om (i) plaaslike EPNs en EPF van vrugteboord grond te isoleer en identifiseer; (ii) die patogenisiteit van inheemse EPN en EPF isolate teen die derde larwe stadium van Medvlieg te evalueer onder optimum laboratorium toestande en van die effektiefste isolate te selekteer en te toets in 'n meer natuurlike (sand) omgewing; (iii) te bepaal watter konsentrasie benodig word om 50% mortaliteit (LD_{50}) te veroorsaak in die *C. capitata* populasie deur die gebruik van geselekteerde isolate; en (iv) 'n opname van wespe, wat parasiete is van vrugtevlieë, in die Wes-Kaap, Mpumalanga en Limpopo provinsies te identifiseer. Die versameling van grondmonsters het 'n groot opbrengs van inheemse entomopatogeniese isolate voortgebring, insluitende EPNs met nuwe bakteriële assosiasies. Die EPF, *Metarhizium robertsii* (MJ06), was ook geïsoleer direk vanuit 'n Medvlieg larwe. Tydens die bepaling van die graad van vatbaarheid van die derde larwe stadium van die Medvlieg vir EPN infeksie (100 IJs / 50 µl), is daar bevind dat al die EPNs hoogs effektief teen die vlieg-pes is, maar met laer konsentrasie (50 IJs / 50 µl), was *Heterorhabditis noenieputensis* die effektiefste EPN. Laasgenoemde spesie, sowel as *Steinernema yirgalemense*, wat tans in die proses is om geformuleer te word vir kommersiële gebruik, het verdere toetse ondergaan deur die gebruik van 'n sand bioassessering-sisteem. *Heterorhabditis noenieputensis* het 'n wesenlike hoër mortaliteit (94-100%) getoon, en alhoewel die meeste Medvlieg larwes papies geword het, was die meerderheid geïnfekteer met nematodes. *Steinernema yirgalemense* het effektiewe beheer getoon met 58-79% van die larwes wat as volwassenes doodgegaan het. Die LD_{50} van *H. noenieputensis* was 37 IJs / insek, wat 14 keer meer effektief was as *S. yirgalemense*. Plaaslike EPF isolate en kommersiële produkte is teen die derde instar larwes getoets deur gebruik te maak van die dip metode. Al die EPF isolate was patogenies en het sigbare infeksie (mikose) van 57-74% veroorsaak. Verlaagde humiditeit het mikose laat daal, en veroorsaak dat die inheemse isolaat, MJ06, die hoogste mikose van 55% getoon het. Derde instar larwes is by sand gevoeg en behandel met die EPF, *M. robertsii* (MJ06) en *Beauveria bassiana* (6756) (wat gedurende die plaaslike grond opname geïsoleer was), het as volwassenes doodgegaan en mikose ondergaan (62-86%). Parasitiese wespe was deur vrugte versameling gevind, maar weens uitdagings van lae DNA konsentrasies, versterkings en beperkte beskikbaarheid van die strepie-kodes van Suid-Afrikaanse wespe wat parasities is tot vrugtevlieë, het spesies identifisering ingeperk. Die gebruik van sentinel lokvalle – wat die uiteensetting van appels wat infesteer is met Medvlieg eiers, larwes en papies is – het geen wespe gelok gedurende die studie nie, maar voorsien 'n eenvoudige en goedkoop metode om van gebruik te maak in toekomstige studies. Die studie dokumenteer potensiële EPN (*H. noenieputensis* SF669) en 'n EPF (*M. robertsii* MJ06) kandidate, wat effektiewelik beheer toon van die grond stadia van Medvlieg, en kan in toekomstige studies 'n fokuspunt as biologiese beheermiddels gebruik word. Die meesterstudie voorsien nuwe opwindende navorsing op potensiële biologiese beheermaatreëls wat gebruik kan word in 'n geïntegreerde plaagbeheerprogram, om die Mediterreense vrugtevlieg op 'n volhoubare en doeltreffende manier te bestuur.

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CHAPTER 1

An overview of the Mediterranean fruit fly and potential of local entomopathogens and parasitoids for use as biological control agents

Introduction

The fruit industry in South Africa is of significant economic and social importance, providing 1.34 permanent jobs per hectare and contributing extensively to the country's agricultural exports and foreign income with an annual turnover of R13.63 billion (Hortgro, 2016). Insect pests remain one of the most significant threats to the industry, with fruit flies (Diptera: Tephritidae) being a key pest, as they attack a wide range of commercially produced fruits and vegetables (Ekesi et al., 2016). These tephritid fruit flies cause direct damage and crop loss and furthermore are of quarantine importance thus, causing restriction of access to lucrative export markets (White and Elson-Harris, 1992). The control of these pests is pertinent to the horticultural industry and the country, and should be controlled in an Integrated Pest Management (IPM) approach, which is eco-friendly, sustainable and effective.

Mediterranean fruit fly

Distribution

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) also known as the Medfly, is one of the most notorious fruit fly pests of global economic importance, as it causes major fruit losses, requires costly control measures and restricts global fruit trade because of its class A2 quarantine pest status (White and Elson-Harris, 1992). Although this species has a southeast tropical, sub-Saharan Africa origin (De Meyer et al., 2002; Headrick and Goeden, 1996), it has become widespread throughout the tropical and warm temperate regions of the world (Malacrida et al., 2007). Although a tropical fruit fly species, it has a wider tolerance for cooler temperatures, thus allowing it to become more widespread than other such species (Nyamukondiwa and Terblanche, 2009; Thomas et al., 2010). It was introduced into Australia, Hawaii, tropical areas of America, the Mediterranean Region and its many islands, making it the most widely distributed tephritid pest to date (White and Elson-Harris, 1992). In South Africa, it is a prevalent pest across the country (Du Toit, 1998), and it has been present in the Western Cape Province since the late nineteenth century (Annecke and Moran, 1982).

Host range

The Medfly is a polyphagous pest attacking more than 400 host plant species (Capinera, 2001; Copeland et al., 2002). In Africa, it has been recorded from over 100 fruit types and is the continent's most polyphagous tephritid (Virgilio et al., 2014). Commercial hosts include apples (*Malus pumila*),

pears (*Pyrus spp.*), plums (*Prunus domestica*), mangoes (*Mangifera indica*), apricots (*Prunus armeniaca*), guavas (*Psidium guajava*) and citrus fruits. Fecund females generally prefer to attack thin-skinned, ripe and succulent fruits (Thomas et al., 2010). This pest is also able to complete its development in vegetables, such as peppers and eggplant, but is not considered a serious vegetable pest (Capinera, 2001). The Medfly also attacks and utilizes a wide variety of indigenous non-commercial plants, and this plays an important role in its distribution and prevalence in South Africa (De Villiers et al., 2013; Grové et al., 2017). Because many of its' hosts are commercially important in South African local and export markets, the threat of this pest to the economy is potentially enormous.

Life cycle

Adult females become sexually mature and start laying eggs within 5-10 days after emergence. The females lay up to 20 eggs per fruit and can produce 300-1000 eggs in a lifetime (McDonald and McInnis, 1985). The females are easily recognized by their distinct ovipositor, which is long and pointed (Picker et al., 2004). During oviposition, female flies use their ovipositor to pierce and lay their eggs below the skin of fleshy ripening fruit, wherein their eggs hatch after 3-10 days and the larvae develop by feeding on the fruit pulp (Figure 1.1; Kapongo et al., 2007; White and Elson-Harris, 1992). Ripening fruit that are firmer and not yet juicy are preferred as they provide for easy oviposition and prevent the drowning of the eggs and larvae (Thomas et al., 2010).

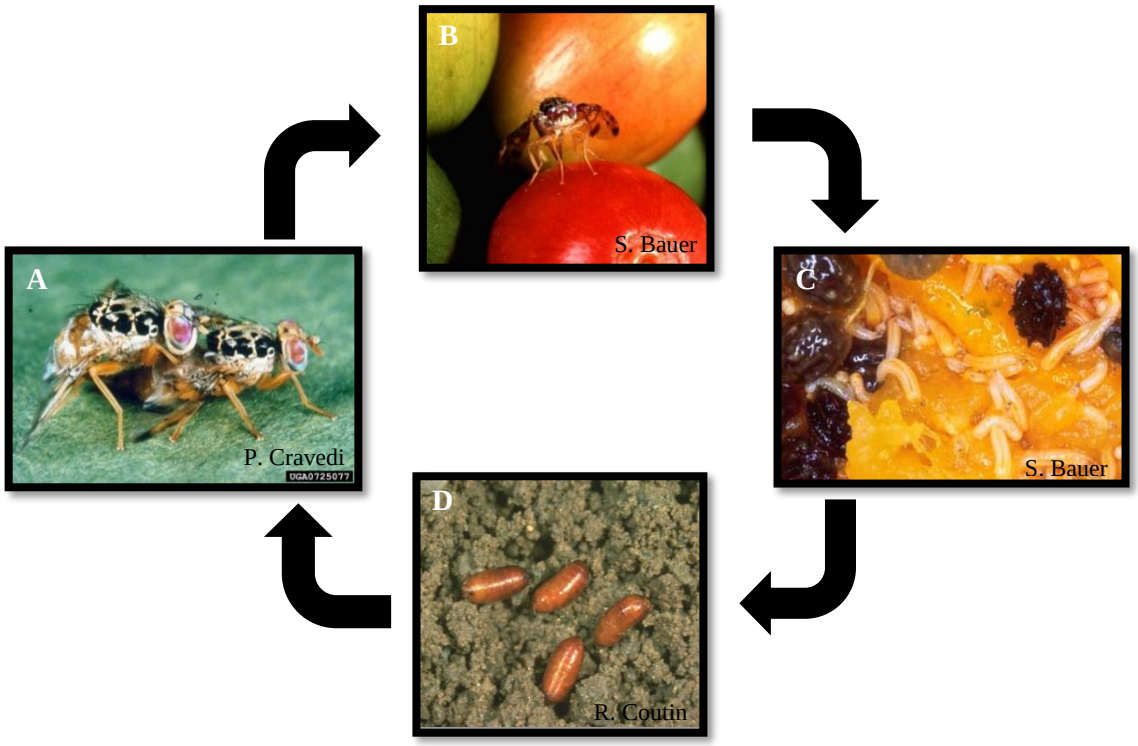

Figure 1.1 Images depicting the life cycle of *Ceratitis capitata*: mating (A); female adult ovipositing eggs (B); larval development within the host fruit (C); pupation in the soil (D).

The offspring go through three instars before becoming fully developed. The third instar larvae exit the infested fruit by 'jumping' out and pupating, within 2-12 hours, just below the soil surface. Medfly larval development can take between 6 and 26 days, depending on temperature and fruit type (Thomas et al., 2010). Adults emerge from the puparium after 8 to 40 days (Annecke and Moran, 1982; White and Elson-Harris, 1992). Newly emerged flies crawl to the surface before searching for mates, usually from 6 – 8 days after eclosion, to start reproduction and continue to infest more fruit. Their life cycle can be completed in 3-4 weeks (about 25 days) if eggs are oviposited in ripe fruit and conditions are warm. The ability of third instar larvae and pupae to get into direct contact with the soil provides an opportunity to utilize biological control agents such as soil-dwelling entomopathogens in fruit fly IPM. When this is integrated with parasitoid wasps that may attack fruit fly eggs or larvae still in the fruit, it can provide a control strategy targeting all immature stages of the Medfly for more effective control.

Damage

Oviposition by the female fruit fly (Figure 1.2), and the resultant larval feeding, causes direct damage to the fruit. When the female punctures the fruit with her ovipositor, secondary organisms may take advantage of these punctures to spread infection that cause the attacked host to start rotting and eventually drop, even if no eggs were deposited (Kapongo et al., 2007). Depending on the host fruit, discolouration may occur on the surface of the fruit where eggs are laid (Virgilio et al., 2014). This is problematic, as even the smallest blemishes will result in quality loss and be unsuitable for local and international markets that demand high quality, appearance-pleasing produce (Stibick, 2004).

Figure 1.2 Two female Mediterranean fruit flies (*Ceratitis capitata*) preparing to lay eggs in an apple.

Although the burrowing and feeding of the developing larvae in the fruit can result in costly direct crop damage and loss (White and Elson-Harris, 1992), the indirect loss can be more substantial due to quarantine restrictions on export markets, particularly countries where this pest is exotic, to prevent invasion and establishment in their fruit producing areas (Badii et al., 2015). *Ceratitis capitata* is a quarantine pest in the USA, China, and New Zealand, and of concern in Japan (Virgilio et al., 2014).

Control strategies

In order to manage fruit fly pests, eradication or an integrated pest management (IPM) approach is recommended (Ekesi et al., 2016). Eradication of the target pest using area-wide approaches to create fruit fly free zones is ideal, but high established populations of the pest make this strategy nearly impossible, and is thus, only possible early on in an invasion. Where a pest has already established, the focus should be on suppressing the population, and this is best done through deploying an IPM strategy. The IPM approach involves utilising a combination of management techniques to control the targeted pest (Ekesi et al., 2016; Kogan et al., 1999).

Chemical control

Chemical pesticides are still a widely used strategy in fruit fly management as they give fast and effective results (Dolinski and Lacey, 2007). For example, the spraying of an organophosphate insecticide called Diazinon is still widely used to control fruit fly larvae and pupae in the soil (Ekesi et al., 2002). Insecticides may also be used in conjunction with the bait application technique, which involves spraying of an attractant such as HymLure protein hydrolysate mixed with an insecticide (Mercaptothion or GF-120 spinosad) on a weekly or fortnightly basis (Manrakhan and Addison, 2014). An attract and kill strategy (i.e. with parapheromones or protein food bait) also utilises insecticides to kill adult fruit flies in a localized trap. This technique deploys bait stations which attracts and kill males and/or females (Ware et al., 2003); and is used in the male annihilation technique where only males are trapped using the lure combined with an insecticide (Ekesi, 2016).

Pesticides, however, are damaging to the environment; can harm non-target species; pests may build up resistance towards them and they can lose their persistence resulting in the need for repeated applications (Ekesi et al., 2002; Wong et al., 1992). Certain export regulations also restrict the use of insecticides on certain commercial fruits (e.g. citrus), further highlighting the need for alternatives (Barnes et al., 2015). The negative effect of insecticides on natural enemies of fruit flies is specifically alarming as these organisms are important for the natural suppression of these pests (Adan et al., 2011). The complete elimination of pesticides is not realistic, but exploring and implementing alternatives which are environmentally-friendly and sustainable can reduce the volume of pesticides required for overall control.

Sterile insect technique (SIT)

The sterile insect technique (SIT) is a method being applied in the Western Cape Province, South Africa, which involves the mass release of irradiated sterile male Medflies. They mate with wild females which then lay infertile eggs, resulting in a decrease of the wild population (Barnes et al., 2015). This 'insect birth control' programme, which began in South Africa in the late 1990s, is already a more environmentally-friendly way in managing this pest. However, it has had varying success rates and is not a stand-alone approach, thus still requiring additional methods to assist in the control of the Medfly (Barnes et al., 2015).

Orchard sanitation

In this cultural control method, all fallen fruits are collected and destroyed (Ekesi et al., 2007). This is done to either prevent fallen fruit from providing an easy host for new infestations by the resident flies in the orchard, or to destroy any eggs or larvae that may be developing within the fallen fruit. Use of an augmentorium is recommended as it allows parasitoids to escape, but will prevent adult flies from escaping back into the orchard (Klungness et al., 2005). Although orchard sanitation is regarded as the first line of defence against Medfly, it is often neglected due to high labour inputs and costs (Barnes and Venter, 2006), with resultant negative consequences, as it allows the population to sustain itself into the next season.

Biological control

The non-target nature of insecticides, pesticide residue level restrictions, resistance build-up and negative environmental impacts necessitates the need to identify alternative control methods for Medfly (Calvitti et al., 2002). An environmentally-friendly and sustainable alternative to pesticides is the use of natural enemies to control insect pests, better known as biological control agents, which come in the form of pathogens, parasitoids and predators (Dolinski and Lacey, 2007). Entomopathogens, such as nematodes and fungi, are particularly valuable in that they are highly species specific; can effectively be incorporated with methods such as SIT and softer pesticides; are safe for the environment, beneficial insects, consumers and applicators; and they can be applied just before harvest (Dolinski and Lacey, 2007). Parasitoid wasps are similarly of great value as they are self-dispersing, not harmful to human health and have been used elsewhere, for example in Australia, to effectively control fruit fly pests (Spinner et al., 2011).

Globally, many biological control programmes aimed at fruit flies have been implemented and proven to significantly lower the populations of Medfly (Wharton, 1989). The search for natural enemies of the Medfly started as early as 1902, when George Compere travelled the world to find suitable parasitoids and predators to ship back to Australia (Wharton, 1989). Since then, many classical and augmentative biological control programmes have been implemented and incorporated into IPM

systems. Identifying locally occurring natural enemies of the Medfly is important as these would be better suited to controlling the pest, largely because they are already adapted to the local natural ecosystem (Malan and Hatting, 2015). However, in South Africa, there have only been a few studies aimed at investigating biological control agents effective against the Medfly, and thus further research into these alternatives is required.

Entomopathogenic nematodes

Biology

Certain species of nematodes are entomopathogenic ('pathogenic to insects') and most entomopathogenic nematodes (EPNs) belong to the families Heterorhabditidae and Steinernematidae. The mutualistic bacteria within the gut of the nematodes are crucial when it comes to killing the host. Bacteria associated with steinernematids belong to the genus *Xenorhabdus* and those associated with heterorhabditids belong to the genus *Photorhabdus* (Lewis et al., 2015).

Infective juveniles (IJs), also known as dauers, are the free-living, stress-resistant stage of the nematode life cycle that vector bacteria to infect insect hosts (Stock, 2015). When the IJs find a suitable insect host, they enter through natural body openings such as the mouth, anus or spiracles (Figure 1.3). Thin areas on the cuticle of the host may also be a point of entry, especially by heterorhabditids as they possess a dorsal tooth (Griffin et al., 2005). Once the IJs enter the insect host, they move to the haemolymph to release symbiotic bacteria which reproduce and release toxins, causing death of the insect pest within 48 hours (Stock, 2015). The nematodes are able to grow and develop into adults within the cadaver for 1-3 generations, while feeding on bacteria and the insect host tissue.

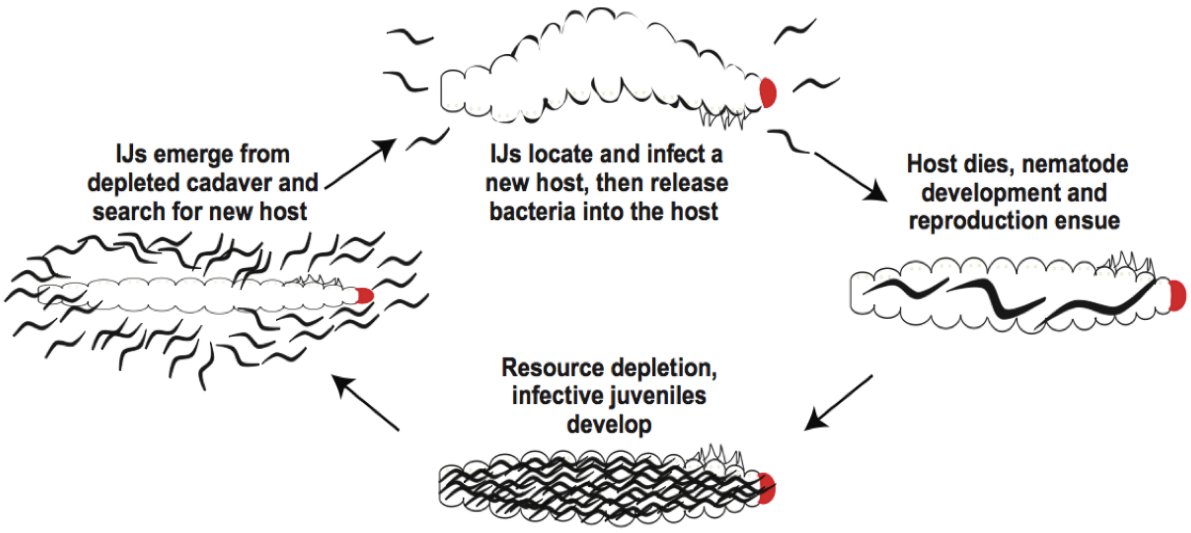

Figure 1.3 Lifecycle of entomopathogenic nematodes (EPNs) in an insect. (A. Dillman)

The first adult generation of all *Heterorhabditis* species are hermaphroditic (self-fertilizing females), while the second generation has males and females. This differs from most *Steinernema* species where all the generations have both males and females that need to mate in order to propagate (Stock, 2015). When food starts depleting a new progeny of IJs, carrying symbiotic bacteria in their intestines, abandon the host cadaver in search of new hosts (Stock, 2015). This free-living stage of EPNs is mobile and can actively forage for new potential hosts, thus sustaining the nematode life cycle (Lewis et al., 1992). The IJs are also very persistent and can live in the soil for several month till they find a suitable host as a food source (Poinar, 1990).

Surveying for EPNs

For biological control studies, it is important that native strains are documented from the intended area of use to avoid biological pollution and potential negative impacts of deploying exotic species. Using indigenous species for controlling local insect pests is expected to be more suitable, as they are better adapted to the local environment (Hiltpold, 2015; Piedra-Buena et al., 2015). Native nematodes are more likely to be effective, without undesirable effects, and would also be more persistent in the soil which may reduce the amount of applications required (Griffin 2015). In South Africa, several known and new species of *Heterorhabditis* and *Steinernema* have recently been isolated and described (Malan and Hatting, 2015; Malan and Ferreira, 2017). These were isolated from soil samples taken from a variety of habitats across several Provinces in the country (South Africa) (Table 1.1).

Table 1.1 Occurrence of *Heterorhabditis* and *Steinernema* spp. in different Provinces and habitats throughout South Africa (taken from Malan and Ferreira, 2017)

Species	Authority	Province	Habitat	Reference
H. bacteriophora	Poinar, 1976	Eastern Cape, KwaZulu-Natal, Mpumalanga, Western Cape	Various habitats	Grenier et al., 1996; Malan et al., 2006, 2011; De Waal, 2008; Hatting et al., 2009
H. noenieputensis[a]	Malan, Knoetze and Tiedt, 2012	Eastern Cape, Northern Cape	Citrus, fig	Malan et al., 2014
H. safricana[a]	Malan, Nguyen, De Waal and Tiedt, 2008	Western Cape	Natural vegetation, peach	Malan et al., 2006; De Waal, 2008
H. zealandica	Poinar, 1990	Eastern Cape, Northern Cape, North West, Mpumalanga, Western Cape	Citrus, natural vegetation	Malan et al., 2006, 2011; Molotsane et al., 2007; De Waal, 2008.
S. citrae[a]	Stokwe, Malan, Nguyen, Knoetze and Tiedt, 2011	Western Cape	Citrus	Stokwe et al., 2011
S. fabii[a]	Abate, Malan, Tiedt, Wingfield, Slippers and Hurley, 2016	Mpumalanga	Black wattle	Abate et al., 2016
S. innovation[a]	Çimen, Lee, Hatting, Hazir and Stock, 2014	Free State	Grain field	Çimen et al., 2014a
S. jeffreyense[a]	Malan, Nguyen, and Tiedt, 2015	Eastern Cape	Guava	Malan et al., 2015
S. khoisanae[a]	Nguyen, Malan and Gozel, 2006	Western Cape	Apple, citrus, grapevine, grass, grassland, natural vegetation, rooibos	Malan et al., 2006, 2011; Molotsane et al., 2007; De Waal, 2008; Hatting et al., 2009
S. nguyeni[a]	Malan, Knoetze and Tiedt, 2016	Western Cape	Fynbos	Malan et al., 2016
S. sacchari[a]	Ntengha, Knoetze, Berry and Tiedt, 2014	KwaZulu- Natal	Sugarcane	Nthenga et al., 2014
S. tophus[a]	Çimen, Lee, Hatting, Hazir and Stock, 2014	Western Cape	Grapevine	Çimen et al., 2014b
S. yirgalemense	Nguyen, Tesfamariam, Gozel, Gaugler and Adams, 2004	Mpumalanga	Citrus	Malan et al., 2011

[a]Type specimen

Native EPNs are collected by taking soil samples and baiting the EPNs with susceptible insects, such as greater wax moth larvae (*Galleria mellonella* Linnaeus [Lepidoptera: Phyralidae]) or mealworms (*Tenebrio molitor* Linnaeus [Coleoptera: Tenebrionidae]). After a week of exposure to the soil, dead larvae are placed on modified White's traps (Kaya and Stock 1997; Figure 1.4) and IJs are harvested within the first week of emergence (Malan et al., 2006). The 2006 study by Malan et al. involved surveying for EPNs from 498 soil samples, taken from areas in the southwest parts of South Africa. After baiting with wax moth larvae, nematodes were isolated from 7% of the samples. The dominant genus isolated was *Heterorhabditis*, while *H. bacteriophora* (Poinar) was the most common species isolated. This study was the first to record *Heterorhabditis zealandica* (Poinar) in South Africa. In another study, a total of 202 soil samples were collected from citrus orchards in the Western Cape, Eastern Cape and Mpumalanga, and from these, 17% yielded EPNs (Malan et al., 2011). Similarly, it was found that majority (89%) of the isolates were heterorhabiditids and the nematode species *H. bacteriophora* was dominant in citrus orchards (Malan et al., 2011). This study was the first to report *Steinernema yirgalemense* (Mráček, Tesfmariam, Gozel, Gaugler and Adams) from South Africa.

Figure 1.4 A modified White's trap of dead *Galleria mellonella* (wax moth) larvae infected with entomopathogenic nematodes on a Petri dish, lined with moist filter paper in a larger glass Petri dish containing distilled water.

Previous research using EPNs to control fruit flies

Screening for the pathogenicity of EPNs against various fruit fly species has been performed in regions all across the world (Table 1.2). Species of the genera *Anastrepha* (Schiner), *Bactrocera* (Macquart), *Dacus* (Fabricius) and *Rhagoletis* (Loew) have been tested and EPNs have shown to be promising against tephritid pests. For example, two steinernematids, *Steinernema carpocapsae* (Weiser, 1955) (Wouts, Mráček, Gerdin and Bedding) and *Steinernema feltiae* (Filipjev) (Wouts,

Mráček, Gerdin and Bedding) were highly pathogenic against the larvae of *Rhagoletis indifferens* (Curran)and further showed great persistence in different soil types, highlighting their potential as effective biological control agents (Yee and Lacey, 2003). There have also been several studies on efficacy of EPNs on *Ceratitis capitata* (Wiedemann). One of the first studies that showed the potential for the use of EPNs to suppress Medfly populations was by Poinar and Hislop (1981), who looked at mortality of adult *C. capitata* caused by parasitic nematodes. Their findings led to further investigations on other Medfly life stages, different nematode strains as well as using EPNs against other fruit fly species.

Several studies also found that EPNs were unable to infect the pupal stage of different fruit fly species (Beavers and Calkins, 1984; Karagoz et al., 2009; Lindegren and Vail, 1986; Malan and Manrakhan, 2009; Soliman, 2007; Yee and Lacey, 2003). However, some studies had different conclusions regarding pupal susceptibility. A study by Barbosa-Negrisoli et al. (2009) carried out on the pre-pupae and pupae of *A. fraterculus* showed both stages to be infected by numerous nematode strains. However, they defined pre-pupae as larvae leaving the fruit with a 'jumping habit' and pupae as those in the process of 'integument sclerotization'. Their conclusions can thus be related to other studies, which used mature third instars and early stage pupae, as these stages do not yet possess a fully sclerotized puparium. A more recent study testing various EPNs against 8- and 14- day old *Bactrocera zonata* (Saunders) and *C. capitata* pupae found a mean mortality between 40-60% for both pupal ages (Nouh and Hussein, 2014). These conclusions contradict the study by Soliman (2007) who investigated the susceptibility of *B. zonata* and *C. capitata* pupae to *Steinernema riobrave* (Cabanillas, Poinar and Raulston) and *H. bacteriophora* at ages 1, 3, 6, 8, 12, 24 hours and 8 days old, and found no mortality of pupae occurring after they were older than 8 hours. Studies by Yee and Lacey (2003), Karagoz et al. (2009) and Malan and Manrakhan (2009) found no susceptibility of pupae, which were between 1 day and 3 weeks old, to the respective EPNs species they tested. The age of the pupae tested is clearly an important consideration, as nematodes may still be able to infect the pupae before the puparium is completely sclerotized or formed. Because nematodes were found in the puparia of Western Cherry fruit flies (*R. indifferens* (Curran)) after 7 days of exposure, Patterson Stark and Lacey (1999) investigated possible modes of entry into the puparia based on where the IJs were found. The heterorhabditids they tested were found clustering near the mouth and anus while the steinernematids were generally found adhering to the spiracles or posterior end of the puparia. Nonetheless, there is no literature about any openings on the puparium of fruit flies, nor on the degree to which natural openings of pupariating larvae close.

One of the most widely tested EPNs is *H. bacteriophora.* Several studies have used different strains of this species, collected from different parts of the world and have found it to be pathogenic against multiple fruit fly species, including *Anastrepha ludens* (Loew) (Toledo et al. 2005; 2006), *Ceratitis rosa* (Karsch) and *C. capitata* (Malan and Manrakhan, 2009) (Figure 1.5). This nematode is a

widespread species commonly present in South Africa (Malan et al. 2006; Hatting et al., 2009; Malan et al., 2011) and other African countries including Kenya, Cameroon, Egypt and Ethiopia (Malan and Hatting 2015). Many researchers have performed studies using only *Steinernema* species, particularly *S. carpocapsae, S. feltiae* and *S. riobrave* (Gazit et al., 2000; Lezama-Gutiérrez et al., 2006; Yee and Lacey, 2003). These studies have mainly been conducted in the laboratory using Petri dishes, various sized cups or multi-well plates. These have been done mostly by placing the fruit fly host onto moistened filter paper or soil, and inoculating with a nematode suspension (Malan and Manrakhan, 2009).

Figure 1.5 A dead third instar *Ceratitis capitata* larva infected with entomopathogenic nematodes (EPNs).

Different EPN species can vary in their host range and thus specificity. Some have been found to be very specific, such as *Steinernema scapterisci* (Nguyen and Smart), which mainly attacks adult mole crickets (Lu et al., 2017), while others are able to attack a wider range of insect pests, such as *S. feltiae* (Piedra-Buena et al., 2015). High host specificity may reduce the potential negative effects on non-target organisms, but a broad host range is more desirable for marketing of a commercial product (Piedra-Buena et al., 2015). In South Africa, government legislation regulates EPN-based products under the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act 36 of 1947. This is because such products are deemed an 'agricultural remedy', and commercialisation is thus a slow and lengthy process (Malan and Hatting, 2015). EPNs have the potential to be successful biological control agents or biopesticides as they have a wide host range, pose no harm to the environment or beneficial species, and are effective against many pests living in the soil (Ferreira and Malan, 2014). The only EPN product currently registered in South Africa is Cryptonem. This product is imported from e-nema, a commercial company in Germany, and is based on *H. bacteriophora* (RiverBioscience Ltd). It is registered for the control of soil stages of false codling moth, as well as weevils and white grubs, but has not yet been tested against fruit flies.

Within South Africa, the potential use of EPNs as a biological control agent has previously been tested against many important agricultural pests. For example, several isolates of EPNs from South African soils were pathogenic against the fifth instar larvae and pupae of the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Malan et al., 2011; Malan and Moore, 2016). De Waal (2008) and Odendaal (2015) conducted field tests and found local EPN species to be effective against codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae), under specific conditions. The susceptibility of larvae and adults of the banded fruit weevil, *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae) was also tested, and they were susceptible to several *Heterorhabditis* isolates (Ferreira and Malan, 2014). Therefore, if the soil-life stages of fruit flies are also susceptible to these local EPNs, they are more likely to be adopted by growers as an alternate management method.

The only study that tested EPNs against fruit flies in South Africa was carried out by Malan and Manrakhan (2009). The two researchers conducted preliminary tests on pupariating larvae, pupae and adults of *C. capitata* and *C. rosa* using local strains of *S. khoisanae* (Nguyen, Malan and Gozel), *H. zealandica* and *H. bacteriophora.* These tests were conducted in the laboratory in 24-well bioassay plates using a concentration of 200 IJ/50 µL of filtered water. Their results showed no pupal infection, but both the third instar larvae and adults were susceptible to EPN infection from all tested nematodes. These findings highlighted the potential that EPNs can provide for control of fruit fly pests. Additional studies are required to elaborate these findings further on Medfly as the fruit fly host and the screening of more local EPNs also becomes paramount. Because fruit flies and several pests, including damaging Lepidoptera, spend part of their life cycle in the soil, a control approach based on EPNs could be widely effective and would enhance their acceptance and uptake by growers.

Table 1.2 A summary of previous studies testing EPNs against various tephritid fruit fly species. The number of *Heterorhabditis* (*Hetero.*) and *Steinernema* (*Steiner.*) isolates tested; fruit fly life stage targeted; type of experiment conducted, as well as the relevant reference is provided.

Fruit fly species	EPN isolates		Life stage targeted			Test conducted in		Reference
	Hetero.	*Steiner.*	Larva	Pupa	Adult	Lab	Field	
Anastrepha suspensa	2	4	x	x	x	x	-	Beavers and Calkins, 1984
Ceratitis capitata; Dacus curcubitae; Dacus dorsalis	0	1	x	x	x	x	-	Lindegren and Vail, 1986
Ceratitis capitata	0	1	x	-	-		x	Lindegren et al., 1990
Rhagoletis indifferens	2	3	x	-	-	x	-	Patterson Stark and Lacey, 1999
Ceratitis capitata	6	6	x	-	-	x	-	Gazit et al., 2000
Rhagoletis indifferens	0	3	x	x	x	x	x	Yee and Lacey, 2003
Anastrepha ludens	1	0	x	-	-	x	x	Toledo, 2005
Anastrepha ludens	1	0	x	-	-		x	Toledo, 2006a
Anastrepha serpentina	1	0	x	-	-	x	-	Toledo, 2006b
Rhagoletis cerasi	0	1	x	-	-		x	Herz et al., 2006
Anastrepha ludens	0	2	x	-	-	x	x	Lezama-Gutierrez et al., 2006
Dacus ciliatus	3	2	x	x	-	x	-	Hussein, 2006
Bactrocera zonata	0	1	x	x	-	x	-	Mahmoud and Osman, 2007
Bactrocera zonata; Ceratitis capitata	1	1	x	x	x	x	-	Soliman, 2007
Anastrepha fraterculus	7	12	x	x	-	x	x	Barbosa-Negrisoli et al., 2009
Dacus ciliates	1	1	x	x	x	x	-	Kamali, 2009
Ceratitis capitata	2	3	x	x	-	x	-	Karagoz et al., 2009
Ceratitis capitata; Ceratitis rosa	4	1	x	x	x	x	-	Malan and Manrakhan, 2009
Bactrocera oleae	0	3	x	-	-	x	-	Sirjani et al., 2009
Ceratitis capitata	1	1	x	-	-	x	-	Rohde et al., 2010
Bactrocera zonata; Ceratitis capitata	1	1	x	x	-	x	-	Soliman et al., 2014
Bactrocera tryoni	1	2	x	x	-	x	-	Langford et al., 2014
Bactrocera zonata; Ceratitis capitata	1	1	x	x	-	x	x	Nouh and Hussein, 2014
Rhagoletis cerasi	2	2	x	-	-	x	-	Kepenecki et al., 2015

Entomopathogenic Fungi

Entomopathogenic fungi (EPF), unlike other microbial control organisms, have the advantage of being able to infect an insect host by penetrating the integument and also do not require the host to ingest the fungus for infection to occur (Beris et al., 2013; Dimbi et al., 2003). Because the soil is less affected by extreme environmental conditions it provides a favourable fungal habitat, thus making use of EPF a sustainable strategy in the control of the soil stages (mature larvae and pupae) of tephritid fruit flies (Quesada-Moraga et al., 2006). Furthermore, the low environmental impact and minimal risk of EPF to other non-target arthropods makes them a prime candidate as an alternative control (Ekesi et al., 2005; Inglis et al., 2012). Of all pathogens, fungi have one of the widest arthropod host ranges. Species such as *Beauveria bassiana* (Balsamo) (Vuillemin) (Hypocreales: Cordycipitaceae) and *Metarhizium anisopliae* (Metschn.) (Sorokin) (Hypocreales: Clavicipitaceae) are able to use a wide variety of insect hosts from several orders. However, it is now well understood that individual isolates within these common species may be highly specific and have restricted host ranges (Wraight et al., 2007). Therefore, the use of EPF as a biological control agent against important pests such as the Medfly requires further exploration, especially in South Africa.

Biology

EPF are distributed globally and use a wide range of insects as hosts (Sookar et al., 2008). The majority of EPF belong to the Hypocreales order of the Ascomycota phylum and commonly belong to the following entomopathogenic genera: *Aspergillus* Micheli, *Beauveria* Balsamo, *Culicinomyces*, *Hirsutella* Patouillard, *Metarhizium* Metschnikoff, *Nomuraea* Yasuda, *Isaria* (=*Paecilomyces*) Samson, *Tolypocladium* Gams and *Lecanicillium* (=*Verticillium*) Gams and Zare (Inglis et al., 2001).

All EPF have a life cycle consisting of a parasitic stage in which they infect the host causing death, followed by a saprophytic phase after the hosts' death (Augustyniuk-Kram and Kram, 2012). EPF produce asexual spores, known as conidia, which adhere to the cuticle of the host and produce a germ tube which penetrates the integument into the hosts' haemocoel (Figure 1.6). After the fungi overcome the immune defences of the host and causing mortality, the fungi continue to grow vegetatively within the host by forming hyphal bodies or blastospores. Eventually the EPF exit as hyphae, via saprophytic outgrowth, and produce more conidia (Inglis et al., 2001). Some EPF species, such as those belonging to the genera *Metarhizium* and *Beauveria*, produce powerful toxins that kill the pest relatively fast (Inglis et al., 2001). The unique ability of EPF to infect insects through their cuticle makes them good potential biological control agents for larvae and pupae in the soil, but also for adult fruit flies (Dimbi et al., 2003).

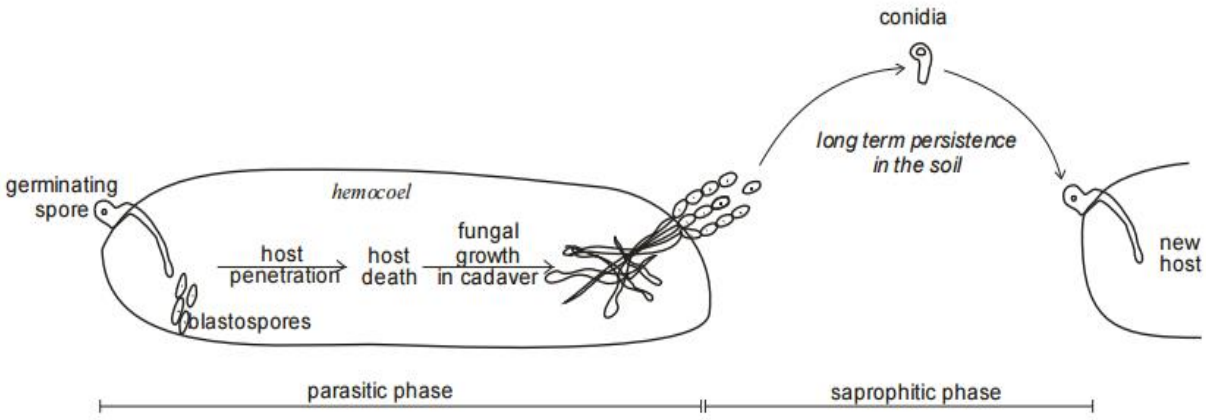

Figure 1.6 The lifecycle of entomopathogenic fungi from the order Hypocreales (Augustyniuk-Kram and Kram 2012).

Previous research on using EPF to control fruit flies

Fungi are diverse, widespread, have extensive host range and the EPF species often cause epizootics, resulting in the natural control of insect populations (Wraight et al., 2007). The nature of these soil-occurring microbial organisms has led to much research on their pathogenicity against certain pest insects. More specifically, most of the attention has been focused on entomopathogenic fungal species from the genera *Beauveria* and *Metarhizium* as these are diverse, do not leave residues and have good potential for commercialization (Ali, 2014).

Surveying for native EPF isolates in local soil samples is an important first step in identifying a suitable biological control agent. In Mauritius, a survey for local EPF found that all three species isolated were pathogenic to two important fruit fly pests, *B. zonata* and *Bactrocera cucurbitae* (Coquillett) (Sookar et al., 2008). Similarly, in the isolation process of EPNs described in the previous section, EPF can be isolated from soil by baiting with a susceptible insect. *Galleria* larvae have been widely used as bait for EPNs, and are also successfully used to isolate EPF from the soil (Zimmerman, 1986). In South Africa, surveys for local EPF are limited, but there have been promising results that have encouraged the need for further research. For instance, Goble et al. (2010) isolated sixty-two fungal isolates from 288 citrus soil samples collected in the Eastern Cape Province. They made use of the *Galleria* bait method, which obtained the most isolates, but also baited the soil with key citrus pests including Medfly larvae, in order to isolate target-specific EPF. Another survey conducted in the Western Cape Province yielded thirty-nine isolates of EPF, the most common being a new species, *Metarhizium robertsii* (Abaajeh and Nchu, 2015).

The potential of using EPF for control of fruit flies and the increasing need for alternatives to chemical control has resulted in wide-ranging research for their use against tephritid species around the world. These studies have covered several fruit fly genera including *Anastrepha* (De La Rosa et al., 2002; Lezama-Gutiérrez et al., 2000), *Bactrocera* (Carswell et al., 1998; Mahmoud, 2009); *Ceratitis* (Beris et al., 2013; Ekesi et al., 2002) and *Rhagoletis* (Daniel and Wyss, 2009). Most of the research has been conducted in laboratory trials, with only a few field experiments (e.g. Ekesi et al. 2005; 2011). A laboratory study in Australia conducted by Carswell et al. (1998) tested the susceptibility of adult Queensland fruit flies, *Bactrocera tryoni* (Frogatt), as well as another dipteran species, *Musca domestica* L. (Diptera: Muscidae), to a *Metarhizium anisopliae* isolate. Their study found 100% mortality of the adult flies after 7-9 days at high temperatures (25°C and 30°C), with the first deaths observed after 4-5 days (Carswell et al., 1998). A study in Mauritius, tested several native EPF isolates against *B. zonata* and *B. curcubitae* adults and showed high mortalities of up to 98% and 94% mortality, respectively, after 5 days (Sookar et al., 2008). The high efficacy of EPF against adult fruit flies has also been demonstrated for the Mediterranean fruit fly, with some strains resulting in 100% mortality (Castillo et al., 2000; Dimbi et al., 2003).

Studies on the efficacy of EPF on larval and pupal life stages have yielded varying results for different tephritid species. After being dipped into a fungal solution of *B. bassiana* strains for 30 seconds, the larvae and pupae of the Mexican fruit fly, *A. ludens*, showed low (1-8%) larval mortality, and 0% pupal mortality (De La Rosa et al., 2002). The larvae of the European cherry fruit fly, *Rhagoletis cerasi* (Loew), exposed to isolates of *B. bassiana, M. anisopliae* and *Isaria fumosorosea* (Wize), also showed very low susceptibility as none of the isolates induced more than 25% mortality (Daniel and Wyss, 2009). It is important to note that although susceptibility was low, Daniel and Wyss (2009) found that 4.2-20.8% of pupae showing mycosis, which could act as a source of new conidia (and thus new insect infections) in the soil. Studies done on the Medfly and other *Ceratitis* species have shown mixed results. When third instar larvae of *C. capitata* and *C. rosa* var. *fasciventris* (Karsch) were exposed to sand inoculated with isolates of *B. bassiana* and *M. anisopliae*, there was varyingly high pupal (25-94%) and adult (32-94%) mortality (Ekesi et al. 2002; 2005). Similarly, after 4-5 day old *C. capitata* pupae were dipped for 30 seconds in different fungal isolate solutions, 45.6-55.4% died and developed mycelia (Beris et al., 2013). High adult mortality observed was from those that emerged from the treated pupae, although there was variation between the different isolates, with *M. anisopliae* yielding the lowest mortalities.

However, Toledo et al. (2006a) on the other hand reported different strains of *M. anisopliae* and *B. bassiana* to have no effect on larvae or pupae of fruit flies. Furthermore, a study recently conducted in South Africa by Goble et al. (2011) tested native EPF strains, collected in the Eastern Cape during an earlier study, against the soil life stages of *C. capitata, C. rosa* and *T. leucotreta*. Fifteen strains of *B.*

bassiana, five strains of *M. anisopliae* and one strain of *Metarhizium flavoviride* (Gams and Rozsypal) was tested in a soil bioassay. The effect on the soil life stages was minimal as the percentage of pupal mycosis did not exceed 25% and 12% for *C. rosa* and *C. capitata*, respectively. The deferred effect on adult mortality was greater, but no more than 35% mycosis was seen in the Medfly adults (Goble et al., 2011).

Although the mortality found in their study was low, Goble et al. (2011) provided critical initial findings on the susceptibility of Medfly to native fungal strains, which provides crucial baseline data for further exploration in South Africa. It is important to note that all the isolates tested in this study were sampled from citrus orchards or adjacent areas/habitats (Goble et al., 2010). Thus, there is scope for testing of other native species found in different habitats as well as commercial EPF products, not yet tested against *C. capitata.* Furthermore, it is clear that all the life stages are susceptible to EPF and could potentially be used in the control of larvae and/or pupae stages for which there are currently no management measures in South Africa (Malan et al., 2011).

Parasitoid wasps

Many species of Hymenoptera parasitize fruit-infesting tephritids, often attacking them when they are hidden within the fruit as eggs or larvae, or in the soil as pupae (Wharton et al., 2000). These natural enemies have long ovipositors with which they locate the egg, larva or pupa and parasitize it with its' own offspring (Karagoz et al., 2009). Parasitic wasps (or parasitoids) are wasps that actively search for and use insects as hosts to lay their eggs, allowing their larvae to feed and live as internal parasites, eventually emerging as adults and killing the host insect. This makes them an effective natural enemy and potential control agent (Quicke, 1997).

Different parasitoids will have varying lifespans, but a native generalist parasitoid found in South Africa, *Muscidifurax raptor* (Girault and Sanders) (Pteromalidae), is known to have a lifespan of about 21 days and in that time lays between 100-115 eggs (Kapongo et al., 2007). High rates of parasitism can result in the reduction of fruit fly populations, thus providing a natural control of important pests.

Searching for and identifying effective natural enemies is crucial for the successful implementation of biological control programmes. For instance, a study on tephritid parasitoids in Northwestern Argentina, recorded five native larval-pupal parasitoids (Ovruski et al., 2004). However, these native species lacked the ability to parasitize the Medfly, which is an introduced fruit fly for Argentina. This inability of native parasitoids to parasitize invasive species highlights the need for enhancing research efforts to identify more efficacious natural enemies in the aboriginal home of the invasive pest, which could be introduced in a classical biological control programme. However, local and target-specific parasitoids, if present, would offer the greater control as they would be pre-adapted to the habitats and

life strategies of that specific host, and would be especially useful for the control of an important agricultural pest like *C. capitata* (Headrick and Goeden, 1996).

Surveys

Most parasitoid surveys have involved the collection of fruit infested with the target pest and holding the fruit under optimal conditions in a device that would retain any emerging parasitoids. A study in Brazil surveyed for native parasitoids associated with fruit flies by sampling mature fruit from the host tree, as well as fallen fruit. A tephritid species-specific association with a parasitoid was only determined if a single species of fly emerged from the same holding container as the wasp, and no other potential hosts emerged, such as Drosophilidae. From the more than 9000 fruit that they sampled, they were able to identify 461 hymenopterans associated with a particular *Anastrepha* fruit fly species (Flavio et al., 2004). In another study, fruits were dissected to recover fruit fly eggs or to verify the insect hosts when adult parasitoids had emerged (Wharton et al., 2000). This method is simple, but unless the pest is isolated from the fruit, the emerging parasitoids can, at best, only be regarded as associated with the pest. A further drawback with fruit collection is the reduced time that the pest is exposed to parasitoids and it also does not allow for the collection of any pupal-attacking parasitoids. However, the collection of infested fruit remains the easiest and most natural method for sampling fruit flies and their parasitoids and can be useful in explorative studies in areas where parasitoid knowledge is limited.

Other sampling methods stem from research on what attracts parasitoids to their fruit fly hosts. A study focusing on the host searching behaviour of a braconid wasp, *Diachasmimorpha kraussii* (Fullaway), found that although direct larval cues were not important in host selection, the parasitoid had a greater preference for fruit infested with the larvae, which was probably due to chemicals emitted as the larvae feed and move through the fruit (Ero, 2009). The use of sentinel trapping by infesting fruit with the target hosts and setting it out in the field, can thus be a valuable method for sampling species-specific parasitoids. For example, it has been used to sample for *Drosophila*-attacking parasitoids in Barcelona, Spain when strawberries infested with larvae or covered with pupae of *Drosophila suzukii* (Matsumura) were placed in a plastic cup, covered with a net and hung in commercial farms (Gabarra et al., 2015). Rossi Stacconi et al. (2013) also used sentinel trapping by placing larvae of *D. suzukii* and *D. melanogaster* on Petri dishes in red delta traps and hanging them 1-2 m above the ground on crops and surrounding vegetation. The only example of tephritid fruit fly sentinel traps is a study by Eitam and Vargas (2007). They investigated the response of the egg parasitoid *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae) to infested fruit on the ground, tree and in the canopy by using sentinel papayas containing *B. dorsalis* eggs. Single papayas were placed in a cage, with twenty *B. dorsalis* females for 24 hours, allowing them to lay eggs, before being set out as bait in small and large field cages. Their study also found that *F. arisanus* avoided ground fruit

and thus highlighted the importance of sanitation in orchards in order to benefit from natural enemies (Eitam and Vargas, 2007). This can be achieved through the use of an augmentorium which traps and kills adult fruit flies from the fruit, while still allowing parasitoids to escape (Klungness et al., 2005).

A more advanced method has also been used to identify parasitoid-host linkages. Rougerie et al. (2011) used molecular techniques on the gut contents of parasitoids in order to identify their host. They discovered that the DNA of the host could persist in the gut of the parasitoid even after metamorphosis occurred. They were able to successfully use parasitoid gut DNA to confirm parasitoid-host relationships to several Diptera and Lepidoptera. Such 'molecular analyses of parasitoid linkages' (MAPL) can assist with biocontrol agent identifications and provides a more rapid approach for confirming such relationships (Rougerie et al., 2011).

Parasitoids used as biological control agents for fruit flies

The pest status of the *Ceratitis capitata* has been important globally for many decades. Thus, as far back as 1902 a search began in Australia for parasitoids to be used against this species in classical biological control programmes (Billah et al., 2008). Since then, there have been major programmes aimed at deploying a biological control strategy for other fruit fly pests, but complete classical control is yet to be achieved (Wharton, 1989). Wharton (1989) highlights that although complete control is not possible, biological control can play a significant role in the reduction of pest population numbers. On Pacific Ocean islands, for example, parasitoids are widely used to supress species of *Bactrocera.* The egg-attacking parasitoid *F. arisanus* was successfully introduced into French Polynesia in 2002 to control *B. dorsalis,* which had invaded and established in 1996. Similarly, the introduction of this same parasitoid to Hawaii was able to reduce the *B. dorsalis* population by 95% (Vargas et al., 2012), and in Kenya, its' introduction has offered significant control against this invader fruit fly (Mohamed et al., 2010). In Florida, the augmentative release of *Diachasmimorpha longicaudata* (Ashmead) was used to supress the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Sivinski et al., 1996). In the Mediterranean Region, *Psyttalia concolor* (Szepligeti) was used against the olive fruit fly, *Bactrocera oleae* (Rossi) (= *Dacus oleae*), but has also been found to be a natural enemy of *C. capitata* in Kenyan coffee fields (Kimani-Njogu et al., 2001). Another East African parasitoid, the recently discovered *Fopius ceratitivorus* (Wharton), was found to successfully attack Medfly in Hawaii (Bokonon-Ganta et al. 2005; 2007). The many successes involving parasitoids as fruit fly biological control agents emphasize their potential for such use elsewhere in the world.

Despite the importance of parasitoids for naturally supressing pest populations, there is limited information about tephritid fruit fly parasitoids in South Africa. A comprehensive natural enemies list is presented in Stibick (2004), listing 61 parasitoid wasps of Medfly, 23 of which are of African origin, but only three species are present in South Africa. The three species associated with *C.*

capitata are *Opius humilis* (Silvestri), *Trichopria capensi* (Kieffer) and *Biosteres bevisi* (= *Fopius bevisi*) (Brues). In the last 40 years, there have only been three exploratory studies for fruit fly parasitoids in South Africa. The first known study looked at the parasitoids of the olive fruit fly, *B. oleae*, in the Western Cape and Mpumalanga areas, and 25 different species were reared from commercial and wild olives (Neuenschwander, 1982). More recently a survey was carried out on wild olives in the Eastern Cape to gain knowledge on the insects feeding on these fruits, as well as the natural enemies present (Mkize et al., 2008). Two fruit fly species, *B. oleae* and *Bactrocera biguttula* (Bezzi), were the most abundant, and four parasitoid wasps were associated with these species. The only study in South Africa to document parasitoids associated with *C. capitata* was carried out by Manrakhan et al. (2010). They reared four species of fruit fly parasitoids from ripe coffee berries in Mpumalanga, two of which had never before been recorded in South Africa, *Psyttalia perproxima* (Silvestri) (Hymenoptera: Braconidae) and *Tetrastichus giffardianus* (Silvestri) (Hymenoptera: Eulophidae). A complete list of fruit fly associated parasitoids, which have been found in South Africa, is presented in Table 1.3.

Establishing the presence of new or yet unreported parasitoids in South Africa will not only be useful for biological control efforts in this country but also have the potential for redistribution in other places like California, which has a similar climate (Mkize et al., 2008). It is apparent that there is a need for more of such follow-up studies in South Africa to better understand the natural control already in place for pests such as the Medfly. Although most of the parasitoids reported in South Africa are egg-larval parasitoids, one study has also shown the potential of using a generalist pupal parasitoid, *M. raptor*, for Medfly suppression in vineyards (Kapongo et al., 2007). However, this parasitoid is not acclimatized to the vineyard environment, as it is mainly found controlling *M. domestica* near dairy farms and in poultry houses where conditions are warm. Natural enemies already adapted to certain climates and pest life cycles provide the greatest potential as biological control agents (Headrick and Goeden, 1996). Furthermore, *M. raptor* was unable to parasitize Medfly pupae in the soil and thus could not be utilised as a biological control agent (K. Pringle *pers. comm.*).

Table 1.3 Parasitoid species recorded in South Africa, which were associated with several genera (*Ceratitis*, *Dacus*, *Bactrocera*, *Trirhithrum*, *Acanthiophilus*) of fruit flies (Diptera: Tephritidae).

Parasitoid species	Authority	Area	Reared from	Fruit flies present	Reference
Opius africanus	Szépligeti	South Africa	Unknown	*C. rosa* *D. oleae*	Silvestri 1914; Wharton and Gilstrap, 1983
O. dacicida *O. lounsburyi* *Triaspis (Sigalphus) daci* *Bracon celer* *Chrysonotomyia erythraea* *Eupelmus afer* *Halticoptera daci* *Psilus (Galesus) silvestrii* *Cirrospilus variegatus*	Silvestrii Silvestrii Szépligeti Szépligeti Silvestrii Silvestrii Silvestrii Kieffer Masi	South Africa	Unknown	*D. oleae*	Silvestri 1914a/b, 1915
Bracon celer *Opius lounsburyi* *Opius africanus* *Opius dacicida* *Microdontomerus* sp. *Tetrastichus* sp. *Chrysonotomyia erythraea* *Tachinaephagus zealandicus* *Eupelmus urozonus* *Halticoptera daci* *Pteromalus semotus* *Eurytoma oleae* *Sycophila aethiopica* *Eupelmus spermophilus* *Ormyrus* sp.	Szépligeti Silvestrii Szépligeti Silvestrii - - Silvestrii Ashmead Dalman Silvestrii Walker Silvestrii Silvestrii Silvestrii -	Cape Province and Transvaal, South Africa	Cultivated and wild olives	*D. oleae*	Neuenschwander, 1982
Coptera robustior	Silvestrii	South Africa	Unknown	*C. capitata* *C. punctata*	Yoder and Wharton, 2002
Trichopria capensis	Kieffer	South Africa	Unknown	*C. capitata*	Wharton and Gilstrap, 1983; Narayanan and Chawla, 1962

Fopius bevisi	Brues	South Africa		*C. capitata* *T. queritum*	Wharton and Gilstrap, 1983
Opius humilis	Silvestrii	South Africa	Unknown	*B. passiflorae* *C. capitata*	Stibick, 2004 Wharton and Gilstrap, 1983
Opius afreutretae	Wilkinson	South Africa	Unknown	*A. muiri*	Narayanan and Chawla, 1962
Psyttalia concolor *Psyttalia lounsburyi* *Utetes africanus* *Bracon celer*	Szépligeti Silvestrii Szépligeti Szépligeti	Easter Cape, South Africa	Wild olives	*B. oleae* *B. biguttula*	Mkize et al., 2008
Bracon celer *Psyttalia perproxima* *Psyttalia humilis* *Tetrastichus giffardianus*	Szépligeti Silvestrii Silvestrii Silvestrii	Mpumalanga, South Africa	Robusta *cv*. coffee berries (*Coffea canephora*)	*C. capitata*	Manrakhan et al., 2010
Muscidifurax raptor	Girault & Saunders	Commercially produced fly parasitoid South Africa	Unknown	Unknown	Pringle, 2009
Opius species *Fopius* species		Hoedspruit, Limpopo, South Africa	Marula fruit	*C. cosyra*	Moxley, 2016
Opius phaeostigma	Wilkinson	South Africa	Unknown	*D. ciliatus* *D. demmerezi*	Narayanan and Chawla, 1962; Thompson, 1943; Wharton and Gilstrap, 1983

Molecular identification techniques

The use of molecular techniques has become increasingly popular and can aid in accurate species identification. Advances in technology have resulted in non-destructive methods for DNA extraction, thus allowing the preservation of the specimen for morphological assessment and museum deposition (Gilbert et al., 2007). Currently, both morphological and molecular identifications are required or are at least desired, for accurate species identification. The curation of the voucher specimen remains necessary to confirm and support molecular identification and together with ecological information may also increase the "phylogenetic resolution" of the organism (Gariepy 2007).

DNA barcoding is a powerful molecular method, which is strengthened by the universal-nature of many primers and, for some groups, large databases (Jinbo et al., 2011). A gene region is amplified using primers in a Polymerase Chain Reaction (PCR), and sequenced. The standard region used for DNA barcoding is usually cytochrome oxidase I (COI) (Jinbo et al., 2011). Sequences are then compared to available gene sequences on databases such as Genbank and BOLD in order to identify the species. The use of such molecular techniques to support morphological identifications provides an advantage for non-specialists to easily identify specimens. Furthermore, universal primers, such as LCO1490 and HCO2198 or TL2-N-3014 ('Pat') and C1-J-2183 ('Jerry') (Folmer et al., 1994; Simon et al., 1994), are useful when the targeted organism is unknown and could also allow for the identification of new and cryptic species (Gariepy et al., 2007).

Correct identification is important when searching for a biological control agent (Gariepy et al., 2007). It allows for the understanding of the organism's relationships with its host and other species, ensures that the correct species is being mass-reared and released, and prevents the introduction of an inefficient species for control (Hoddle et al., 2015). Molecular tools are useful for identification of smaller insect specimens, such as parasitoid wasps, which can be difficult to identify morphologically, generally requiring expert verification (Jenkins et al., 2012). Other potential biocontrol agents, such as EPNs and EPF, are much smaller microscopic organisms making molecular tools the preferred identification tool. Although identification of many Hypocrealean EPF can be successfully achieved through observation of the asexual morphological characters (Inglis et al., 2001), the increasing complexity of species often requires the use of molecular methods to confirm identification or provide a more robust identification. Cases also exist where morphology may not be sufficient to distinguish between species. For example, the *Metarhizium anisopliae* complex contains multiple species that can only be reliably distinguished using molecular techniques (Bischoff et al., 2009). Similarly, with EPNs, barcoding of genes can provide confirmation of morphological identifications. Use of the primers TW81 and AB28 have been successfully used to amplify the ITS region of both entomopathogenic nematodes and fungi (Hominick et al., 1997).

Biological control agents within an IPM strategy

Biological control agents such as entomopathogens or parasitoid wasps can be more optimally exploited for control of the target pest when incorporated into an overall IPM strategy comprising other proven components. For example, a study carried out in Hawaii showed that releasing the larval-parasitoid, *Diachasmimorpha tryoni* (Cameron) (Hymenoptera: Braconidae), complemented with sterile male *C. capitata* adults, resulted in increased efficiency of Medfly control over time (Wong et al., 1992). This was because the parasitoid focused on attacking the larvae, while the sterile males mated with wild adult females. This increased efficiency was also shown in similar studies against *C. capitata* in Guatemala, as well as for the Caribbean fruit fly, *A. suspensa*, in Florida (Sivinski et al., 1996; 2000). In South Africa, the SIT programme focusing on Medfly is already showing good success rates and additional release of an effective parasitoid could contribute to the overall control of this pest.

EPF can also be incorporated into SIT programmes by inoculating the sterile males with fungal conidia to facilitate and increase EPF transmission and ultimately increase mortality (Toledo et al., 2006a). It has been shown that horizontal transmission of the EPF can occur during mating, thus assisting the spread of the lethal fungal spores. Observations by Dimbi et al. (2009) found males to be undeterred by EPF-infected females and they would even mate with dying or dead females. This could further extend the spread of EPF to wild fruit fly males. A drawback of releasing infected sterile males is that these males have been observed to spend a lot of time grooming themselves, thus delaying their response to or search for females and potentially reducing the efficacy of the approach (Dimbi et al., 2009). Some studies have also shown that certain EPF isolates have no negative effects on non-target tephritid parasitoids and thus these two control methods could potentially complement each other (Ekesi et al. 2005).

The use of EPNs as a biological control can easily be incorporated into an IPM system, as they can be applied using standard equipment, such as sprayers currently used to apply chemical insecticides (Hiltpold, 2015). Their application to the soil can reduce the emergence of adult flies and thus assist in the overall control of fruit flies, by supporting the efforts of SIT programmes. Furthermore, EPNs could be used in conjunction with EPF to increase overall mortality of the target pest. This was found highly successful when the combined effects of selected EPN species together with an isolate of *M. anisopliae* was tested against third-instar larvae of black vine weevil, *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae) under laboratory and greenhouse conditions, causing additive or synergistic interactions resulting in 63-100% mortality (Ansari et al., 2008). A potential issue is the competition between the entomopathogens for a host, which may result in the consequent reduction in their ability to recycle themselves and therefore lower their overall persistence in the soil.

Significance of project

There has been very limited research investigating the use of local EPNs, EPF and parasitoid wasps against fruit flies in South Africa. However, the need for this technology in fruit fly IPM is ever growing as hazardous chemicals are still widely used with the negative effects to the users, environment, natural control agents and consumers becoming of increasing concern. Furthermore, export market requirements specifying which chemicals may be used as well as the minimum level of residue that is allowed is becoming increasingly stringent. This has forced South Africa to look towards alternatives for fruit fly control to protect market access and the economic benefits surrounding the industry. Although several control methods such as the use of bait applications, male annihilation technique and SIT are already employed against *C. capitata*, they target mainly the adult stage of the pest. This project focuses on the control of the soil life stages of this fruit fly and is thus of great importance, as it can complement the IPM strategy already being deployed in South Africa, with higher benefits envisaged. This study will increase the current knowledge on local entomopathogenic species for Medfly control and furthermore seek to expand the knowledge on native fruit-fly parasitoids present in South Africa, for potential use in future biological control programmes.

Aim and objectives

Aim:

To contribute to the knowledge on biological control agents such as EPNs, EPF and parasitoid wasps present in South Africa, which can be used against the Mediterranean fruit fly, *Ceratitis capitata*, and be incorporated into current management practices.

Objectives:

- Isolate and identify native entomopathogenic nematodes and fungi from fruit orchard soils.
- Evaluate the pathogenicity of local EPN and EPF isolates against the third larval instar stage of *C. capitata* under controlled laboratory conditions, and selected species of each in a more natural (sand) environment.
- Estimate the lethal concentration/dose needed to result in 50% *C. capitata* mortality (LD_{50}) using selected EPN isolates.
- Survey for and identify fruit fly parasitoid species occurring in the Western Cape, Mpumalanga and Limpopo Provinces.

NOTE: chapters of this study have been written as separate publishable papers, and for this reason, some repetition across the different chapters has been unavoidable.

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CHAPTER 2

Surveying and screening local entomopathogenic nematodes for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann)

Chapter accepted for publication as 'James, M., Malan, A.P., Addison, P., 2017. Surveying and screening South African entomopathogenic nematodes for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). *Crop Protection*'.

Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), or Medfly, is an important economic pest worldwide. In the Western Cape Province of South Africa, it is regarded as a pest of deciduous fruit, grapes and citrus (Prinsloo and Uys, 2015). Medfly causes extensive economic losses, due to direct crop damage and expensive control methods (including the sterile insect technique), and also restricts access to the export market (White and Elson-Harris, 1992). This species attacks nearly 400 different host plants, and is also multivoltine, producing several generations each year (De Meyer et al., 2002). Direct damage is as a result of female Medfly laying eggs into the fruit. The eggs hatch into larvae that feed and burrow through the fruit, from which they eventually exit out as third instar larvae, to pupate in the soil.

Current control methods aim mainly to control the adults, using insecticidal cover sprays and bait application, as well as the release of sterile males, whereby mating with wild females results in the production of infertile eggs (Barnes et al., 2002; Manrakhan and Addison, 2014). However, the inefficiency or non-target nature of insecticides, residue restrictions, resistance build-up and negative environmental impacts necessitates investigation into alternative control methods (Calvitti et al., 2002).

Organisms that are naturally parasitic on insect pests are known as biological control agents, and can be utilized as environmentally friendly and sustainable control options. Entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae are insect-killing parasites that occur naturally in soils throughout the world (Poinar, 1990). The free-living infective juveniles (IJs) actively move through the soil in search of hosts, which they enter through natural openings, such as the mouth, anus and spiracles. Symbiotic bacteria carried in the gut of the IJ ultimately cause the death of the insect within 48 hours. The IJs continue to feed, develop, and reproduce within the dead host (Griffin et al., 2005). Once the nutrient rich haemolymph has been depleted, a new cohort of IJs exits the dead insect in search of new hosts in the soil (Stock, 2015). Thus, the soil stages of fruit flies, including the third instar larvae, pupae and emerging adults can be targeted as potential hosts for EPNs.

For biocontrol strategies using EPNs, it is important that native strains be isolated in the intended area of use, so as to avoid new biological introductions and the possibility of negative impacts of exotic species on the ecosystem. During the last decade, several known and new species of EPNs have been isolated and described in South Africa (Malan and Hatting, 2015). The recently described EPNs include seven *Heterorhabditis* and eleven *Steinernema* species (Hunt and Nguyen, 2016; Malan and Ferreira, 2017). Of these species, all are endemic to South Africa, except for six species that have previously been reported in other countries (Malan and Ferreira, 2017). Malan et al. (2006) collected 498 soil samples from areas in the south-west parts of South Africa and EPNs were isolated from 7% of these samples. The dominant genus isolated was *Heterorhabditis*, while *H. bacteriophora* (Poinar) was the most common species isolated. This study was the first to record *Heterorhabditis zealandica* (Poinar) in South Africa. In another study, a total of 202 soil samples were collected from citrus orchards in the Western Cape, Eastern Cape and Mpumalanga Provinces, of which 17% yielded EPNs (Malan et al., 2011). Similarly to the previous study, the majority (89%) of isolates detected were heterorhabditids, with the nematode species *H. bacteriophora* being dominant in citrus orchards (Malan et al., 2011). The 2011 study was the first to report the presence of *Steinernema yirgalemense* (Mráček, Tesfamariam, Gozel, Gaugler and Adams) in South Africa.

Several studies have shown the potential of using steinernematid and heterorhabditid species as biological control agents of fruit flies (Gazit et al., 2000; Kamali et al., 2009; Karagoz et al., 2009; Malan and Manrakhan 2009). Most of the studies have focused on the EPN infectivity of the third instar larval stage of species from various tephritid genera, including *Anastrepha, Dacus, Bactrocera, Rhagoletis* and *Ceratitis.* The global impact of the Medfly has prompted numerous research studies to find a suitable EPN for its control (Abbas et al., 2016; Gazit et al., 2000; Karagoz et al., 2009; Langford et al., 2014; Mamhoud et al., 2016; Nouh and Hussein, 2014). However, in South Africa, only one study has been undertaken to date, in which locally occurring EPNs were tested against various *C. capitata* and *Ceratitis rosa* (Karsch) life stages in the laboratory (Malan and Manrakhan, 2009). The promising results from this study justify further investigation using other native species, such as *Heterorhabditis indica* (Poinar, Karunakar and David), *Heterorhabditis noenieputensis* (Malan, Knoetze and Tiedt), *H. bacteriophora*, and *S. yirgalemense*. Inclusion of *S. yirgalemense* in this study is important, as it is currently in the process of being mass-produced for eventual commercialisation, due to the effectiveness of its control against the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Malan and Moore, 2016; Malan et al., 2011). Determining its potential against other pests such as the economically significant Medfly could be important, as its efficiency against multiple hosts would likely promote greater uptake by growers.

The main objective of the current study was therefore to isolate and identify local EPNs from soils in fruit orchards, and to test the pathogenicity of several local EPNs against the third instar larvae of *C. capitata* under laboratory conditions. Local EPNs were isolated from the soil by means of trapping

with two susceptible hosts (wax moth larvae and mealworms), and with fruit fly larvae. The pathogenicity of EPNs against Medfly was assessed by screening the known local EPN species under optimal conditions, followed by the testing of selected virulent species in a soil environment. Concentration assays with IJs were carried out to determine the lethal doses required to cause effective mortality of the third instar larvae.

Materials and Methods

Surveying, isolation and storage of nematodes from the field

Source of insects

Third instar larvae of *C. capitata* were obtained from the Department of Conservation Ecology and Entomology's insectary at Stellenbosch University, where they were reared from eggs at 25 ± 2°C, 16L: 8D. Larvae were reared on diet which contained wheat bran, sugar, brewer's yeast, Nipagin™, water, and hydrochloric acid, while the adults were fed on sugar yeast (3:1) and water (Tanaka et al., 1969). Third instar (7-day-old) larvae were used for the pathogenicity screening tests. *Tenebrio molitor* (Linnaeus) (Coleoptera: Tenebrionidae) (mealworms), after having initially been bought from a local pet shop, were cultured in ventilated plastic containers, filled with fine bran, supplemented with carrots, while *Galleria mellonella* (Linnaeus) (Lepidoptera: Phyralidae) (wax moth) larvae were reared according to the techniques used by Van Zyl and Malan (2015).

Soil surveys

Sixty-eight soil subsamples were collected from different pome, stone, grape and citrus fruit farms in the Western Cape Province, at the farms Denau, Welgevallen, Baldric, Timberlea, Oak Valley, and Fontana, during early 2016 and 2017. An additional twelve subsamples were taken from an organic raisin-producing farm, Carpe Diem, in Upington in the Northern Cape Province. Orchards in fruit fly problem areas were selected, as it was expected that natural enemies would be present where high fruit fly infestations occurred. An alcohol-cleaned hand spade was used to dig up the moist soil about 5-10 cm deep near the fruit tree. Each sample consisted of four subsamples of 250 g of soil, consisting of 50 g of soil sampled from five random trees within each quadrant (Figure 2.1). Thus, a minimum of 1 kg of soil was sampled per orchard (Malan et al., 2011). In the field, each 250 g sample of soil was sieved to remove large pieces of debris and directly placed in a labelled 1 L ice-cream container. While in the field, sampled soil containers were placed in the shade to prevent overheating and dehydration, prior to transportation to the laboratory in a crate lined with ice packs.

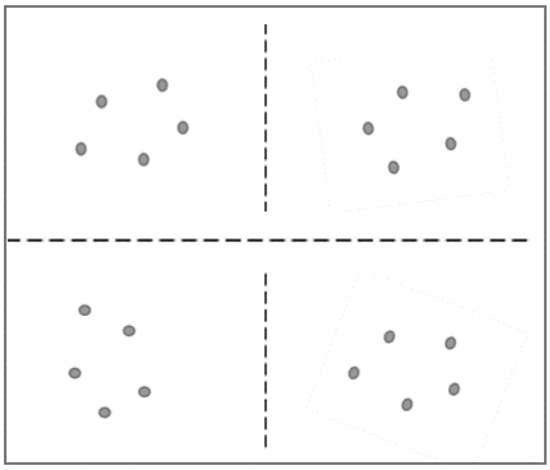

Figure 2.1 Each dot represents a random sample of 50 g of soil collected 5-10 cm deep near the tree base. A total of approximately 1kg of soil was sampled per orchard.

Isolation and storage of nematodes

To maximise EPN isolation, each 250 g subsample was processed separately. If the soil was found to be dry, distilled water was added. Within 5 days of collecting the soil, five *Galleria* larvae and five mealworm larvae were added to each subsample as bait (Van Kleespies et al., 1989; Vanninen et al., 1989). The container was closed with the lid, labelled and stored in a dark growth chamber at 25 ± 2°C. After 5 days each container was inspected and dead, or pupating larvae were removed, while live larvae were left in the soil. The equivalent number of larvae was added to replace those removed. Each dead larva was rinsed with distilled water, surface sterilised by means of dipping it in 70% alcohol, and placed on filter paper in a Petri dish (50 mm diam.). Thereafter, 300 µl of distilled water was added to each dish to provide moisture for any nematode that emerged. Each Petri dish was sealed with Parafilm® and stored at 25 ± 2°C. The process was repeated for two more weeks and soil was thus rebaited twice (Malan et al., 2006, 2011). After a period of 4-5 days, Petri dishes were checked and cadavers that showed symptoms of a potential nematode infection, such as a colour change, were placed on modified White's traps (Kaya and Stock, 1997), at room temperature. The White's traps were monitored daily for IJ emergence, with distilled water being added, if necessary, to prevent the filter paper, and thus the nematodes, from drying out.

After baiting with the more susceptible bait insects, one subsample from each site was sieved, using a smaller size mesh (2 mm), and baited with the Medfly hosts. The method was designed to isolate any Medfly-specific EPNs that might have been present in the soil. Sieving the soil was required to facilitate pupal retrieval. A diet containing second and third instar *C. capitata* larvae was placed on the lid of a 50 mm diameter Petri dish, which, in turn, was placed on top of each soil subsample (Figure 2.2). This allowed the final instar larvae to exit the diet, as they naturally would exit the fruit in the orchard, by jumping out into the soil to burrow and pupate.

After one week, all dead larvae and pupae were sifted from the soil and surface sterilised, as previously described. After separate processing of the insects from each subsample, they were placed on moist filter paper in Petri dishes to allow for nematode development.

To ensure that the nematodes isolated from the bait insects were indeed insect-killing parasites, Koch's postulates for pathogenicity were confirmed (Steyn and Cloete, 1989). Five wax moth larvae were placed on a piece of filter paper in a Petri dish (9 mm diam.), and inoculated with a nematode suspension of approximately 1000 IJ / 800 µl. This was done for all the nematode isolates obtained from the soil samples. All Petri dishes were sealed with Parafilm® and kept in the dark at 25 ± 2°C. After 48 h in the growth chamber, the cadavers were washed and transferred to a clean Petri dish lined with moist filter paper until nematode emergence.

Figure 2.2 Image showing fruit fly diet containing developing *Ceratitis capitata* larvae used as bait for Medfly-specific EPNs that might be present in a soil. The diet is placed on a plate that is positioned on the surface of a soil sample, allowing the larvae to develop naturally, jump out of the Petri dish and burrow into the soil.

EPN identification (extraction, amplification and sequencing)

Nguyen's (2007) technique was followed for DNA extraction as well as for the polymerase chain reaction (PCR) amplification. One young female nematode was placed in 30 µl lysis buffer (500 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20®, 0.1% gelatine, and 3 µl of proteinase K [600 µg / ml]), on the side of a 0.5 µl microcentrifuge tube. After being cut up into pieces with the sharp side of a syringe needle, the tube was frozen for 1 h at -80ºC. The tubes were then incubated in a thermocycler at 65ºC

for 1 h, followed by incubation at 95ºC for 10 min. After centrifugation for 2 min at 11 600 g, the supernatant (20 µl) was transferred to a clean microcentrifuge tube, in which it was stored at -20°C. The primers TW81 (5'- GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'- ATATGCTTAAGTTCAGCGGGT-3'), as suggested by Hominick et al. (1997), were used for amplification of the ITS region. The primers were synthesised by Integrated DNA Technologies Inc. (Coraville, Iowa, USA). Post-PCR purification was undertaken using the NucleoFast® Purification System (Macherey-Nagel). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by the use of electrophoresis on the 3730 × l DNA Analyser (Applied Biosystems) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University).

Pathogenicity of EPNs

Source of EPNs

EPNs were obtained from the collection held by the Department of Conservation Ecology and Entomology, Stellenbosch University, with isolates from a local soil survey also being used (Table 2.1). The IJs of EPNs were cultured *in vivo*, using *Galleria* larvae on modified White's traps approximately 2 weeks prior to screening (Kaya and Stock, 1997). Twenty insect larvae were placed in a Petri dish (90 mm diam.), lined with filter paper and inoculated with 800 µL of the EPN suspension. After 2 days in a growth chamber maintained at 25 ± 2°C, the dead larvae were transferred to clean Petri dishes (Figure 2.3). After 7 to 10 days, the cadavers were placed on modified White's traps, so as to allow for IJ emergence (White, 1927). Emerged IJs were harvested, with the suspension of IJs and distilled water transferred to vented culture flasks daily within a period of 7 to 14 days, and then stored at 12.8°C, before being used for the screening tests within 4 weeks.

Table 2.1 Species name, isolate and source of entomopathogenic nematodes (*Heterorhabditis* and *Steinernema*) tested against third instar *Ceratitis capitata* larvae in pathogenicity tests.

Species	Isolate	Collection/Source	GenBank accession no.	Reference
H. bacteriophora	SF351	Stellenbosch University	FJ4558443	Malan et al., 2006
H. indica	SGS	Stellenbosch University	KU945293	Unpublished
H. noenieputensis	SF669	Stellenbosch University	JN620538	Malan et al., 2014
H. zealandica	MJ2C	Citrus orchard, Hex River Valley	MF370073	Unpublished
S. yirgalemense	157C	Stellenbosch University	EU625295	Malan et al., 2011

Figure 2.3 Wax moth (*Galleria mellonella*) larvae inoculated with different entomopathogenic nematode species in order to culture a fresh batch of each species to be used in the screening tests against *Ceratitis capitata* larvae.

Screening of local EPNs

The pathogenicity of each EPN isolate on *C. capitata* third instar larvae was tested at a concentration of 100 IJs / 50 µl per fruit fly larva. The EPN species used for screening are indicated in Table 2.1. The required nematode concentration was determined as described by Navon and Ascher (2000).

For each EPN isolate, the pathogenicity screening was carried out in 12 alternate wells of a 24-well bioassay plate to reduce possible IJ movement between the wells. Filter paper (12.7 mm diam.) was added to each of the 12 wells before an Eppendorf® micropipette was used to inoculate each well with 50 µL of the adjusted IJ suspension. One third instar *C. capitata* larva was added to each inoculated well, before covering it with a layer of tissue paper with a glass plate on top, to limit the movement of jumping larvae between wells, and then securing the container with a rubber band (Figure 2.4). Five replicate bioassay plates (n = 60) were inoculated in the same manner, and stored in 2 L plastic containers lined with moist paper towels to ensure high humidity. An identical replicate control (n = 60) was prepared on each day of the screening, to which only 50 µL of distilled water was added. All the plates in the 2 L containers were stored in a growth chamber at 25 ± 2°C for 48 h.

Pathogenicity was recorded by means of assessing the mortality caused by the nematode infection of each species, as well as in the control, after 48 h. After rinsing with distilled water, dead insects were transferred to clean Petri dishes (90 mm diam.) lined with filter paper and moistened with 800 µL of distilled water, before being returned to the dark growth chamber for nematode development. After a further 48 h for the small EPNs (500-800 µm) and 24 h for the larger EPNs (>1000 µm), the number of IJs that penetrated each larva was assessed by means of dissecting the host and by counting the

number of IJs present inside the cadaver using a stereomicroscope. The counts were also used to determine the percentage penetration achieved by dividing the number of IJs counted by the concentration used (i.e. 100 IJs / larva). Where the amount of time involved did not allow for immediate dissection, the hosts were frozen, and penetration was assessed at a later stage. The experiment was repeated using a fresh batch of IJs and insects on a later date.

Due to the lack of significant differences between the different species tested, the experiment was repeated using a lower concentration of 50 IJs /50 µl (50 IJs / larva). The second experiment was repeated twice, using fresh batches of IJs and insects, on different dates. For both the nematode concentrations used, the number of IJs of each EPN that penetrated the larvae was counted by means of dissecting the insect larvae.

Figure 2.4 Third instar *Ceratitis capitata* larvae in a single well of a 24-well bioassay plate on filter paper that had been inoculated with infective juveniles.

Sand bioassays

Sand bioassays were performed using two promising EPN species. *Heterorhabditis noenieputensis*, which is an endemic species only found in South Africa, was selected for further testing, due to its high virulence found during the initial screenings (Malan et al., 2014). *Steinernema yirgalemense* was also selected, as it is currently being formulated into a commercial product, thus warranting investigation of its potential use against multiple pests (Ferreira et al., 2016). Sterilised Malmesbury river sand (100 ml) was placed in each 250 ml plastic container, to which 10 ml of distilled water was added. A concentration of 2 000 IJs / 500 µL (21 IJs / cm^2; 200 IJs / insect) was added to each container for each nematode species treatment, while only 500 µL of distilled water was added to the control treatment. There were 10 replicates of each treatment and all containers were covered and

stored at 25 ± 2°C. After 24 h, 10 third instar *C. capitata* larvae were added to each container, and allowed to burrow naturally into the sand (Figure 2.5). The containers were then returned to the 25 ± 2°C growth chamber for a period of 14 days. After 2 weeks, mortality due to EPN was assessed by means of dissecting all dead larvae, pupae, and emerged adult flies. The experiment was repeated with a fresh batch of nematodes and insects on a different test date.

Figure 2.5 Ten third instar *Ceratitis capitata* larvae on sterilized sand that 24 hours prior, had been inoculated with an infective juvenile (IJ) suspension of 2 000 IJ/500 µl.

Lethal concentration tests

To determine the lethal concentration of *H. noenieputensis* and *S. yirgalemense*, different nematode concentrations of each were used. Five concentrations (50, 35, 24, 17 and 12 IJs / 50 µL) were prepared for *H. noenieputensis*, including a distilled water-only control. Concentration tests for *S. yirgalemense* started with a higher concentration, due to it being less virulent against Medfly larvae (200, 100, 50, 25 and 12 IJs / 50 µl). The same procedure was used as for the initial screening, using 12 wells in a 24-well bioassay plate. The mortality of the larvae due to EPN was evaluated after 2 days in the 25 ± 2°C growth chamber. Each larva and pupa was dissected, and only noted as being dead if the presence of nematodes was visually confirmed. Natural deaths in the control were also recorded.

Statistical analyses

In order to identify the most virulent EPN species, the results were analysed using a two-way analysis of variance (ANOVA) to compare the percentage mortality caused by the different EPN species across the two batches (repeats). The analysis was done for data at a concentration of 100 IJs / 50 µl, and again at 50 IJs / 50 µl. A Least Significant Difference, and, in some cases, a Bonferroni, post-hoc test was used to identify differences between the species at each concentration. If the residuals were non-normal, non-parametric analyses using Kruskal-Wallis tests were used, per batch, to confirm the ANOVA results. Data of the penetration by IJs into the third instar larvae were analysed with a one-way ANOVA and a Bonferroni post-hoc test. Prior to the undertaking of the sand bioassay analyses, Abbott's correction formula was applied to the data, to account for the natural deaths that were found to have occurred in the controls (Abbott, 1925). To assess the virulence of EPNs in a sand environment, the Abbott's corrected percentage mortality data were subjected to a two-way ANOVA, followed by a post-hoc test, to identify the significant differences among the mean mortalities. All analyses were performed using STATISTICA 13.0 (StatSoft Inc., 2016). Probit analyses were carried out on mortality data caused by different concentrations, using Polo PC (LeOra Software, 1987).

Results

Soil surveying

From a total of 80 subsamples of soil collected, three EPN species were found in 15 of subsamples (18.75%) (Table 2.2). The species identified included *H. zealandica,* of which the associated bacteria turned the *Galleria*-infected larvae either red or greenish. The other species found was *H. bacteriophora* which caused infected *Galleria*-larvae to turn red. A free-living nematode species, *Oscheius myriophilus* (Poinar), was also isolated. No *Steinernema* species were found during the study and no EPNs were isolated when using *C. capitata* larvae as the bait insect.

Table 2.2 Entomopathogenic nematodes isolated and identified from soil samples taken in the Western and Northern Cape Provinces, South Africa between January 2016 and February 2017.

EPN species	Colour	Strain	Fruit	Farm	Area	GPS	GenBank accession no.
H. bacteriophora	Red	UP2A1	Vineyards	Carpe Diem	Upington	S28° 27'32.67" E21° 19'43.47"	MF372924
H. bacteriophora	Red	UP2A2	Vineyards	Carpe Diem	Upington	S28° 27'32.67" E21° 19'43.47"	MF033536
H. bacteriophora	Red	UP3A1	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372925
H. bacteriophora	Red	UP3A2	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372926
H. bacteriophora	Red	UP3A3	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372928
H. bacteriophora	Red	UP3B1	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372927
H. bacteriophora	Red	UP3B2	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372929
H. zealandica	Green	MJ1C	Table grapes	Denau	Hex River Valley	S33° 30'56.10" E19° 33'10.80"	MF167295
H. zealandica	Red	MJ1B2	Table grapes	Denau	Hex River Valley	S33° 30'56.10" E19° 33'10.80"	MF185663
H. zealandica	Red	MJ2D.2	Citrus	Denau	Hex River Valley	S33° 31'13.90" E19° 32'57.20"	MF370071
H. zealandica	Red	MJ2D.1	Citrus	Denau	Hex River Valley	S33° 31'13.90" E19° 32'57.20"	MF370072
H. zealandica	Green	MJ2C	Citrus	Denau	Hex River Valley	S33° 31'13.90" E19° 32'57.20"	MF370073
H. zealandica	Red	MJ2B	Citrus	Denau	Hex River Valley	S33° 31'13.90" E19° 32'57.20"	MF370074
Oscheius myriophilus	Blue	UP2Binitial	Vineyards	Carpe Diem	Upington	S28° 27'34.36" E21° 19'36.21"	MF372144

Screening of local EPNs

The results from the two batches inoculated with 100 IJs / 50 µl were significantly different from each other, and, thus, could not be pooled ($F_{(1, 58)} = 7.9068$; df = 1; P = 0.00671). Overall, all EPN species caused similar levels of mortality that did not differ significantly(Figure 2.6). Significant differences were however observed between the treatments and the control ($F_{(5, 58)} = 7.5868$; df = 5; P = 0.00002) (Figure 2.6). *Heterorhabditis noenieputensis* and *H. indica* caused a mean mortality >80% in both experiments. The number of IJs that penetrated the larvae differed significantly between the five EPN species tested ($F_{(4, 20)} = 6.9384$; df = 4; P <0.005). The highest penetration, of 22.1%, was achieved by *S. yirgalemense*, implying that, on average, 22 of the 100 IJs penetrated each larva, while only 4.2% of the 100 *H. bacteriophora* IJs succeeded in penetrating the Medfly larvae (Table 2.3).

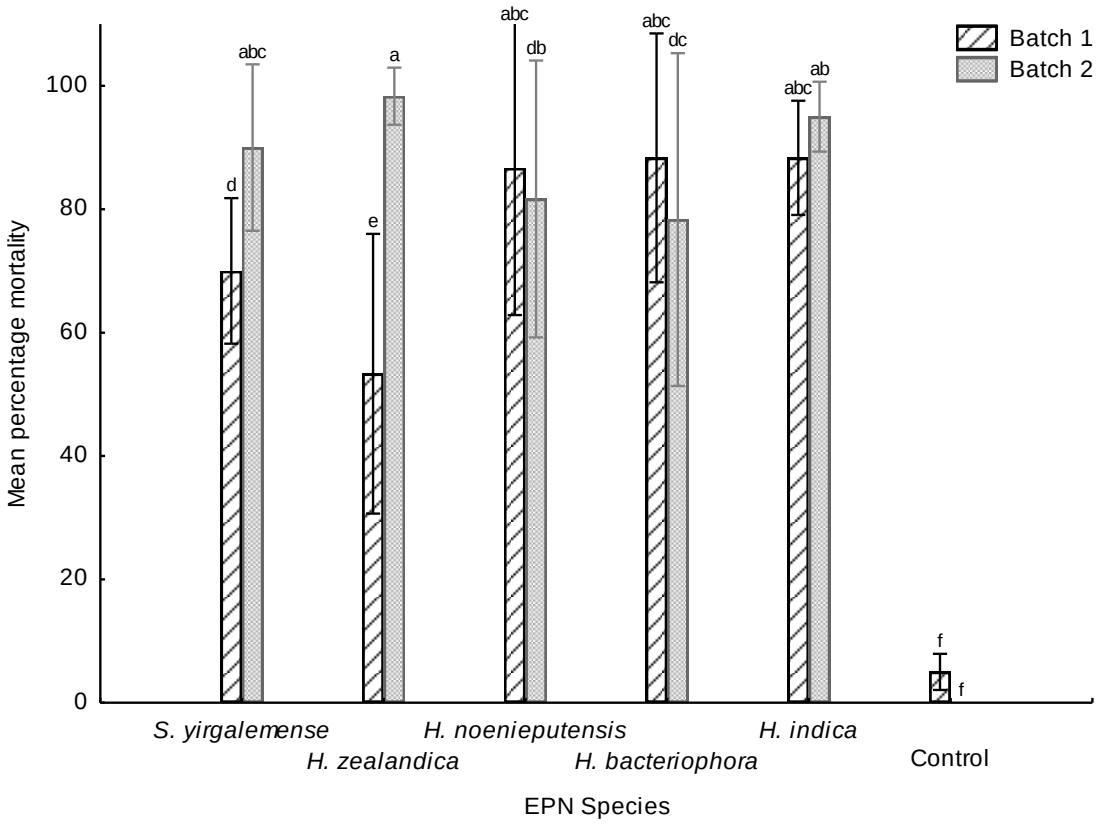

Figure 2.6 Mean percentage mortality (± 95% confidence interval) of the third instar *Ceratitis capitata* larvae caused by the five EPN species tested, using a concentration of 100 IJs / 50 µl, and a water- only control, for the two batches (repeats). Different letters above bars indicate significant differences.

Table 2.3 The mean percentage infective juvenile penetration (± standard error) of each *Steinernema* and *Heterorhabditis* species at two concentrations tested against *Ceratitis capitata* larvae. Different letters indicate significant differences.

Species	**Mean % penetration ± SE**	
	50 IJs / 50 µl	**100 IJs / 50 µl**
H. bacteriophora	2.20 ± 0.56[a]	4.18 ± 0.54[b]
H. indica	0.93 ± 0.29[a]	16.77 ± 1.91[a]
H. noenieputensis	2.07 ± 0.16[a]	13.02 ± 3.18[ab]
H. zealandica	2.00 ± 0.63[a]	11.23 ± 3.65[ab]
S. yirgalemense	2.00 ± 1.19[a]	22.10 ± 2.1[a]

Using 50 IJs / 50 µl, both batches also yielded different results and could not be pooled. In batch 1, *H. noenieputensis* and *H. bacteriophora* caused significantly higher mortality compared to the other species, or the control ($F_{(5, 48)} = 19.733$; df = 5; P = 0.00) (Figure 2.7). In batch 2, *H. indica* and *H. noenieputensis* were the two most virulent species, causing a mean mortality of 96.67% ± 2.045%, and 98.3% ± 1.63%, respectively. In both batches, natural mortality in the control was <5%.

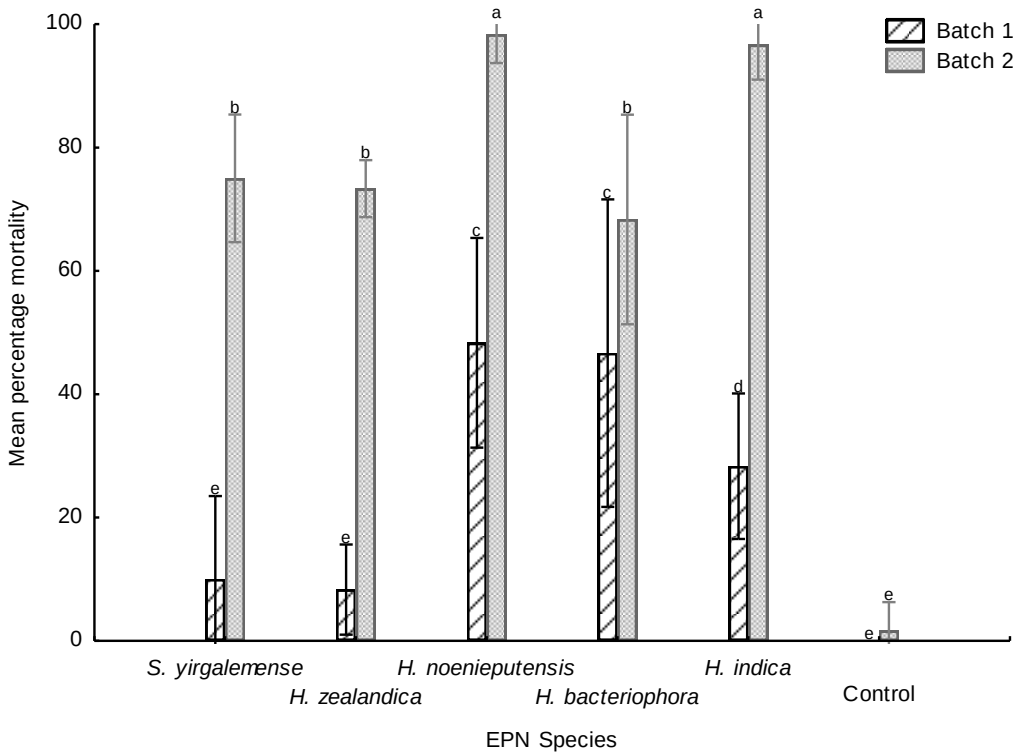

Figure 2.7 Mean percentage mortality (± 95% confidence level) of third instar *Ceratitis capitata* larvae caused by the five EPN species tested, using a concentration of 50 IJs / 50 µl, and a water-only control, for the two batches (repeats). Different letters above bars indicate significant differences.

A higher number of nematodes penetrated the Medfly larvae, when inoculated with 100 IJs, within 48 hours (Table 2.3). *Heterorhabditis indica* had the highest penetration rate (17%), and *H. bacteriophora* the lowest rate (4%), with a mean of only 4 IJs. When the Medfly was inoculated with 50 IJs, the penetration rate of the IJs was low, with no significant difference between the penetration rate of the five EPN species ($F_{(4, 20)} = 0.58815$; df = 4; P = 0.675). Overall, the mean penetration rate was ± 2 % for all species (Table 2.3).

Sand bioassays

The main effects of date and treatment showed a significant difference ($F_{(1, 54)} = 11.038$; P = 0.00161). The mortality caused by *H. noenieputensis* ranged from 93 to 100%, which differed significantly from *S. yirgalemense* (57-74%), and both species showed significant differences (P <0.005) compared with the control (2-20%) (Figure 2.8). The performance of *S. yirgalemense* differed significantly between the two batches; however, in both experiments this species caused over 50% mortality (Figure 2.8). A nematode infected Medfly pupa and adult are shown in Figure 2.9.

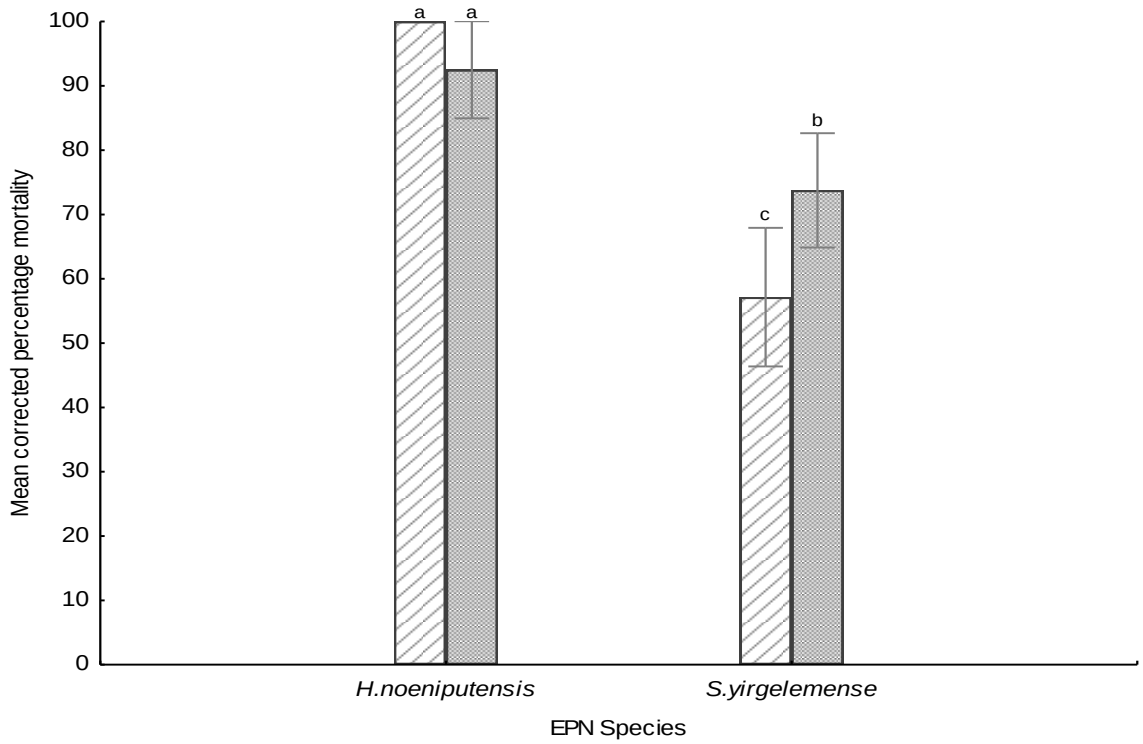

Figure 2.8 Mean corrected percentage mortality (95% confidence level) for the two batches (different test dates) of third instar *Ceratitis capitata* larvae that were exposed in sand inoculated with 2000 IJs, added in 500 µl of water, of *Steinernema yirgalemense* and *Heterorhabditis noenieputensis*. Different letters above bars indicate significant differences.

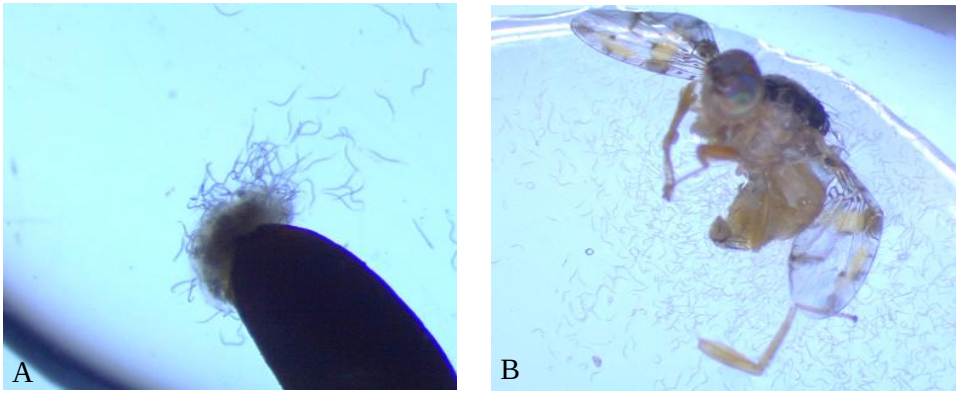

Figure 2.9 IJ emerging from a *Ceratitis capitata* pupa (A) and the abdomen of an adult (B) that were exposed as third instar larvae to sterilized sand that had been inoculated with EPNs 24 hours prior.

In both batches of *H. noenieputensis*, most of the exposed larvae died as infected pupae, whereas, with *S. yirgalemense*, a much lower percentage was infected as pupae (Table 2.4). In contrast, only a few of the larvae died as adults with *H. noenieputensis*, while for *S. yirgalemense*, up to half were infected as adults. Between 2-20% of the larvae died in the controls. In general, few emerging adults were found to be infected within the soil (Table 2.4).

Table 2.4 Number of different life stages of *Ceratitis capitata* infected with entomopathogenic nematodes, after third instar larvae were exposed to 2 000 IJs / insect in 100 ml of sand for 14 days.

Stage infected	*Heterorhabditis noenieputensis* % infection		*Steinernema yirgalemense* % infection	
	Batch 1	Batch 2	Batch 1	Batch 2
Third instar larvae	0	1	0	6
Pupae	99	88	2	37
Emerging adults (in sand)	0	1	3	2
Adults	1	4	53	34
Total	100	94	58	79

Lethal concentration tests

Analysis of the mortality caused at different concentrations using probit analysis, showed that the regression lines for the two species were different (Figure 2.10), as both the slope and the intercept differed (X^2 = 82.7839; df = 2; P <0.001), but were parallel (X^2 = 1.9524; df = 1; P = 0.162). For *S. yirgalemense*, the LD_{50} and LD_{90} were 526.90 and 8863.2 IJs / insect, respectively, whereas, for *H. noenieputensis*, they were 37.80 and 635.84 IJs / insect, respectively (Table 2.5). The relative potency of *H. noenieputensis* was 13.94 times higher than for *S. yirgalemense* (Table 2.5). No natural deaths were observed in the control. An EPN infected Medfly pupa and larva are shown in Figure 2.11.

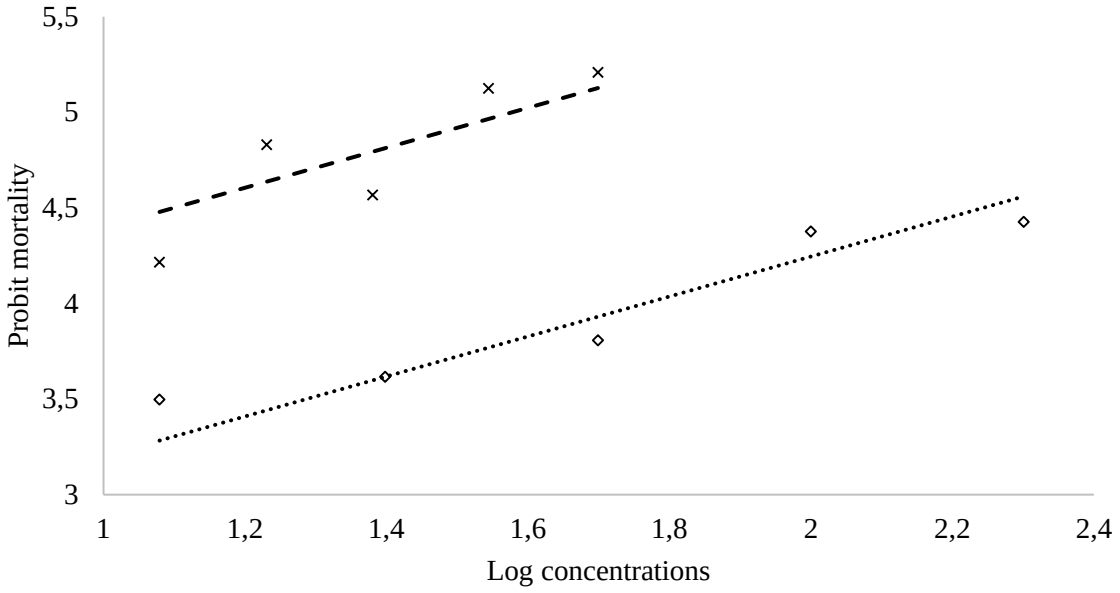

Figure 2.10 Probit mortality obtained at each log concentration tested for *Heterorhabditis noenieputensis* (x) and *Steinernema yirgalemense* (◊) against third instar *Ceratitis capitata* larvae. The regression line formulae for *S. yirgale*mense and *H. noenieputensis* were Y = 2.1546218 +

1.0454295X and Y = 3.3508459 + 1.0454295X, respectively, where X = log (concentration) and Y = probit mortality.

Table 2.5 The lethal dose (LD) of infective juveniles (IJs) of third instar *Ceratitis capitata* larvae, inoculated with different concentrations of *Heterorhabditis noenieputensis* and *Steinernema yirgalemense* in 24-well bioassay plates, with the lower and upper 95% confidence limits.

	H. noenieputensis			*S. yirgalemense*		
	Effective dose	95% confidence limits		Effective dose	95% confidence limits	
	(IJs/insect)	Lower	Upper	(IJs/insect)	Lower	Upper
LD_{50}	37.80	24.54	74.40	526.90	238.42	3818.8
LD_{90}	635.84	210.76	12 714	8863.2	1745.1	765 740
LD_{95}	1415.4	366.77	57 726	19 729.09	3027.05	>666 700

Figure 2.11 A *Ceratitis capitata* pupa and larva that was infected by *Heterorhabditis noenieputensis*. Large first generation female nematodes are visible through the skin of the larva.

Discussion

Part of the Medfly's life stage is spent in the soil; they enter as third instar larvae, pupate, and eventually emerge as an adult fly. This study aimed to isolate and identify any South African EPN species that could potentially be used as biological control agents against *C. capitata*. Screening tests highlighted the pathogenicity of local EPNs against this pest and soil bioassays revealed the ability of selected EPN species to infect *C. capitata* in a more natural soil environment.

No unknown EPN species was isolated, but two known *Heterorhabditis* species were isolated from the areas surveyed. All EPNs isolated from the Hex River Valley in the Western Cape Province were identified as *H. zealandica*, a species first recorded from South Africa in 2006 from Patensie, in the Eastern Cape Province (Malan et al., 2006). Since then, it has also been recorded from the Montagu and Moorreesburg areas of the Western Cape Province (Malan et al., 2011). However, the associated bacteria of the *H. zealandica* isolates from the present study differed from those that were previously isolated from the South African *H. zealandica*, as their infection caused baited wax moth larvae to turn either brick-red or greenish in colour. Such a finding contradicts the 'steel-grey colour' of infected wax moth larvae, as described in an earlier study by Malan et al. (2011). The different colouration of the cadavers is caused by the presence of the symbiotic bacteria carried in the gut of the EPN, which play an important role in killing the host insect. Different isolates containing different bacteria may differ in their virulence against different insect hosts (Stock, 2015). The only other species isolated during the current study was *H. bacteriophora*, which was found in soils on the organic raisin farm in Upington, Northern Cape Province. This was, incidentally, the very first EPN to be described to species level from South Africa by Grenier et al. (1996), and is found to be widely distributed in South Africa, as well as in other African countries (Malan et al., 2006). Both of the EPNs have been shown to cause high mortality against the pupariating larvae and adults of the Medfly (Malan and Manrakhan 2009).

The identification of *Oscheius myriophilus* from the Upington vineyard was significant as this is the first time that this species is reported from South Africa. A study of Swiss isolates of this genus revealed that they are 'facultative kleptoparasites' that scavenge for insect cadavers (Campos-Herrera et al., 2015). The scavengers are referred to as free-living bacterial-feeding nematodes, with their need to feed on and to reproduce within, a dead insect putting them in competition with EPNs.

No EPNs were isolated when soil samples were baited using third instar larvae of *C. capitata*. Goble et al. (2010) baited their soil samples with *C. capitata* and *T. leucotreta* larvae in addition to wax moth larvae, and *C. capitata* attracted a fungal species not isolated from either of the other two species. Although not an EPN, their study showed that the target insect may attract specific entomopathogens not attacking the standard bait insects. Thus, the technique of baiting with various bait insects is regarded as a necessary additional step, as some EPNs may be specific in their choice of

host and may not necessarily cause death in the usual susceptible bait insects. In addition, the technique described in this study can also be regarded as a novel, easy and natural way of getting the third instar larvae of fruit flies to enter into the soil, and should thus be considered in future soil surveys.

EPNs, as obligate parasites, make use of insects as a host to reproduce and breed. This natural process makes the EPNs ideal biological control agents against insect pests, with the identification of highly virulent species, or isolates being an important step in their successful use as biocontrol agents. In the current study, all the EPNs tested were highly pathogenic when they were exposed to *C. capitata* larvae. However, at lower concentrations, some were clearly more virulent than others. The two species that performed the best overall were *H. noenieputensis* and *H. indica*, neither of which had previously been tested against *C. capitata*. Although *H. indica* (KU945293) was isolated in South Africa, it has also been found in soils on several other continents including Asia, Australia and North America (Hominick, 2002). Its virulence against Medfly larvae warrants further investigation of this species as a potential biological control agent. The equally virulent *H. noenieputensis*, which is an EPN species that is endemic to South Africa, has only recently been found and described (Malan et al., 2011, 2014). As both the species are heterorhabditids, they both possess a dorsal tooth that is generally not found in steinernematids, which may have facilitated penetration into the host larvae (Griffin et al., 2005). Their high virulence in these experiments may also be attributed to their relatively smaller body size (± 528 µm), which may have allowed them to more easily penetrate the host through natural openings (Malan et al., 2014). The importance of the size of both the nematode and host insect is highlighted by Bastidas et al. (2014), who found that longer nematode species were less able to infect micro-insects (<5 mm) than shorter ones were. Although third instar *C. capitata* larvae, which are 7-9 mm in length, are not considered micro-insects, the ability of the nematode to complete its life cycle normally might be reduced with an increase in its' body size (Bastidas et al., 2014).

Heterorhabditis bacteriophora SF351 and *H. zealandica* MJ2C, although able to penetrate and kill the pupariating larvae, were less virulent than the smaller heterorhabditids. Both species were previously tested against different life stages of *C. capitata* and found to cause high infectivity of the pupariating larvae (Malan and Manrakhan, 2009). However, these results are not directly comparable to those obtained in the current study, as different isolates were used. This is specifically true for *H. zealandica*, because isolate SF41, as used by Malan and Manrakhan (2009), contains the bacteria *Photorhabdus zealandica* (Ferreira et al., 2014), whereas the isolate MJ2C has an unidentified *Photorhabdus* spp. as its symbiont. This finding highlights the importance of identifying the symbiotic bacteria associated with each EPN, when searching for an appropriate biological control agent (Griffin et al., 2005).

The ability of *S. yirgalemense* to infect and kill Medfly larvae was an important finding in the current study, although the species had a low virulence (10-75%). This species has shown great potential to control the soil life stages of other important pests, including false codling moth, *T. leucotreta* (Malan et al., 2011), and codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) (De Waal, 2008; Odendaal et al., 2015), and is currently undergoing formulation into a commercial product (Ferreira et al., 2016). The pathogenicity of *S. yirgalemense* to Medfly highlights its potential to control multiple pests, which should enhance its acceptability and uptake by growers.

In the initial screening tests, individual Medfly larvae were directly exposed to IJs, while the sand bioassays tested the ability of the different EPNs to locate and infect larvae in a more natural environment. The high infectivity obtained by *H. noenieputensis* in the sand bioassay confirms its potential to control this important pest species. Total infectivity and mortality caused by this isolate was higher than that of *H. bacteriophora* (SF286), which was tested in the same manner against the pupariating larvae of *C. capitata* and *C. rosa* (Malan and Manrakhan, 2009). Importantly, most *C. capitata* died as pupae (88-99%), resulting in no adult flies. However, since Malan and Manrakhan (2009) regarded the pupae as impenetrable, it is likely they were all infected as larvae, allowing them enough time to pupate before death. Other studies also found that various EPNs were unable to infect the pupal stage of different fruit fly species (Karagoz et al., 2009; Lindegren and Vail, 1986; Soliman, 2007; Yee and Lacey, 2003). The above findings suggest that *H. noenieputensis* may be able to find, and infect, the final instar larvae soon after they enter the soil. A potential drawback is the efficiency with which Medflies protect themselves within the puparium. Sclerotisation might affect the emergence of the new IJs, because a hardened cuticle might make it difficult for the IJs to escape, resulting in their death within the puparium. Such a shortcoming would be likely to limit the EPNs' control and require shorter reapplication intervals, and is thus a gap that requires further research.

The infectivity of *S. yirgalemense* (58 to 79%) in the sand was significantly lower than that caused by *H. noenieputensis*. Many were infected as adults, suggesting that the IJs of *S. yirgalemense* mainly attacked the flies as they emerged from the puparium, whereas fewer IJs were able to attack the third instar larvae with success. When the species was tested against FCM larvae, in a similar sand bioassay, it was able to cause 93.5% mortality, with many of the moths also dying as infected adults (Malan et al., 2011). Emerged insects, such as fruit flies infected with *S. yirgalemense*, can potentially fly away from the infected soil, and disperse EPNs over longer distances than was previously thought, if the insects land on moist soil when they die. When pupae of the Western cherry fruit fly [*Rhagoletis indifferens* (Curran)] were exposed to the *Steinernema* species in the soil to assess their effect on adults, relatively low infection rates were found for the emerged adults, ranging from 0 to 53% (Yee and Lacey, 2003). It was suggested that infection probably occurs as the emerging fly crawls to the soil surface. Together with the current findings, it would appear that the period for EPN infection of

fruit flies is the short amount of time that they tend to spend in the soil, as third instars prior to pupation, and the time taken to emerge from the soil.

For the two tested EPN species, the parallel probit regression lines obtained showed a positive relationship, for both species, with the percentage mortality of larvae increasing as the IJ concentration increased. After 2 days, the LD_{90} value for *H. noenieputensis* was 635 IJs / insect, while, for *S. yirgalemense*, a dose of 8863 IJs / insect was required to cause 90% mortality. The former species is, thus, 13 times more potent as a biological control agent against the third instar larvae of the Medfly. However, when various IJ concentrations of another heterorhabditid, *H. zealandica* SF41, were tested against pupating codling moth, an LD_{90} of 275 IJs / ml was obtained within 2 days (De Waal, 2008). A similar LD_{90} value (278 IJs / ml) was also found after 4 days when the same isolate was tested against the banded fruit weevil (Ferreira and Malan, 2014). These findings suggest that the larvae of the Medfly are less susceptible than those of codling moth, and that a higher concentration of nematodes would be required to effectively cause mortality in multiple pests, including Medfly.

In the current study, new isolates of *H. zealandica* were identified, with possibly two new bacterial associations, from commercial orchards in the Hex River Valley in the Western Cape Province, South Africa. Identification of the new strains demonstrates the vast variety of EPN isolates that are present in local soils, and future research should include soil surveying, where appropriate, to determine if other unreported species exist in the country. The present study also revealed the potential of using the local EPN *H. noenieputensis* (SF669) against third instar Medfly larvae entering the soil to pupate. It is recommended that this species be further investigated as a biological control agent for the Medfly, within an integrated pest management programme in South African orchards. Further research should investigate the efficacy of *H. noenieputensis* in field applications, as well as determine the necessary conditions required when applying the nematodes. The current study also highlights the potential of *S. yirgalemense* (157C) as a biological control agent against the Medfly. This species should be exploited for control of Medfly by attacking the adults as they emerge from puparia in the soil. *Steinernema yirgalemense* has already been found to be pathogenic to several other pests, making it an important EPN that could aid in the control of multiple important pests. This study provides important initial findings on suitable EPN candidates for use as biological control agents of the Mediterranean fruit fly.

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CHAPTER 3

Surveying and screening local entomopathogenic fungi for the control of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann)

Introduction

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) or Medfly, is an agricultural pest of economic importance, that is established in many tropical and warm temperate regions of the world, including South Africa (Du Toit, 1998; White and Elson-Harris, 1992). This fruit fly species requires host fruits for the protection and development of its offspring, which are oviposited as eggs below the skin of fleshy, ripening fruits. The eggs develop into third instar larvae before exiting the fruit to pupate in the soil, eventually emerging as adult flies after 6-13 days (Thomas et al., 2010). The larval stages of this pest cause extensive direct crop damage and loss and the associated quarantine status of *C. capitata* places restrictions on access to export markets (White and Elson-Harris, 1992).

The main control strategies employed against *C. capitata* target the adult flies to reduce mating, oviposition and ultimate growth of the population. This is done through the use of chemical insecticide sprays, bait stations which combine protein baits with insecticides, and more recently, the Sterile Insect Technique (SIT) (Barnes et al., 2015; Manrakhan and Addison, 2014). However, problems associated with chemical resistance and the detrimental effects to the environment necessitate the development of alternative methods to enhance the control of this pest, and to sustain and retain export markets.

Entomopathogenic fungi (EPF) are globally distributed soil microbes, which are able to exploit a wide range of insects as hosts and kill them in the process (Sookar et al., 2008). The majority of EPF belong to the Hypocreales of the order Ascomycota, and species known to be pathogenic to fruit flies belong to genera such as *Beauveria* (Balsamo), *Metarhizium* (Metschnikoff) and *Isaria* (=*Paecilomyces*) (Samson), all of which are relatively easy to mass-produce (Dimbi et al., 2003; Ekesi et al., 2007; Inglis et al., 2001). EPF produce conidia, or asexual spores, which, once attached to the integument of an insect, are able to penetrate into the hosts' haemocoel, causing death. Within the cadaver the fungi grow vegetatively, eventually exiting as hyphae that produce new conidia (Inglis et al., 2001). The unique ability of EPF to infect insects through their cuticle makes them effective potential biological control agents for fruit fly larvae and pupae present in the soil, but also for emerging and emerged adults (Dimbi et al., 2003). Their efficacy may be affected by various factors including overcoming the host insects' defences as well as environmental factors (Goble, 2011). The latter includes abiotic factors such as temperature, rainfall, wind and relative humidity. Humidity particularly influences the moisture in the environment, which is important for survival and

development of conidia and could thus influence virulence of EPF against insect hosts (Inglis et al., 2001).

Globally, there has been extensive research on the pathogenicity of EPF against various fruit fly pest species, including the genus *Ceratitis* (Beris et al., 2013; Castillo et al., 2000; Mochi et al., 2006; Quesada-Moraga et al., 2006). Most studies focused on local isolates and the majority of the successes documented on emerging or fully developed adults. However, a strategy targeting the soil life stages of fruit flies, which includes the third instar larvae, pupae and emerging flies, could be important and can easily be incorporated into and enhance an overall Integrated Pest Management (IPM) system, such as that which is used in South Africa against *C. capitata*. However, in South Africa there has to date only been one study assessing the use of local EPF as a potential control against the Medfly and Cape fruit fly, *Ceratitis quilicii* (De Meyer, Mwatawala and Virgilio), previously known as Natal fruit fly (*C. rosa* Karsh) (De Meyer et al., 2016). Third instar larvae were added to EPF-inoculated sand and mortality and visible mycosis of subsequent pupae and adults was monitored. Their study did not find native isolates to cause significant mortality of the soil life stages, but mortality and fungal mycosis were seen in the emerged adults (Goble et al., 2011). Interactions between a fruit fly host and EPF that leads to infection can vary between species as well as between isolates against different insect hosts (Castrillo et al., 2005). The success reported for local isolates in other studies warrants further investigation especially as Goble's study only tested EPF found in the Eastern Cape Province. Furthermore, screening of commercially available products registered against other agricultural pests is also important, as the ability of the product to control multiple pests will enhance its uptake by growers and encourage the incorporation of biological control agents in fruit fly IPM.

Isolation and identification of locally occurring and adapted EPF is another important step in the selection of a virulent agent for use in biological control (Ravensberg, 2010). In South Africa, only a few exploratory soil surveys have been conducted in search of effective insect pathogenic fungal isolates. In one known exploration, sixty-two isolates were sampled from citrus orchards in the Eastern Cape Province (Goble et al., 2010) while sampling efforts in the Western Cape Province yielded thirty-nine fungal isolates (Abaajeh and Nchu, 2015), suggesting that there is a substantial abundance of EPF isolates present in South African soils. Several of the isolates sampled by Goble et al. (2010) have been found to be highly virulent against important agricultural pests highlighting the potential of using local EPF as a method of pest control.

The main objective of this study, therefore, was to isolate and identify local EPF from fruit orchard soils in the Western Cape Province and test EPF pathogenicity against third instar larvae of *C. capitata*. Pathogenicity was assessed by screening commercially available EPF formulated products, as well as locally isolated species, in controlled optimal conditions, whereby third instar Medfly larvae were directly exposed to EPF. The influence of moisture on the virulence of different EPF

isolates was also investigated. These bioassays were followed by testing the virulence of the non-commercial isolates in more natural sand bioassays, in which third instar larvae were added to sand sprayed with an EPF suspension.

Materials and Methods

Surveying, isolation and storage of fungi from the field

Source of insects

Third instar larvae of *C. capitata* were obtained from the insectary at the Department of Conservation Ecology and Entomolog, Stellenbosch University, where they were reared at 25 ± 2°C, 16L: 8D for 7 days prior to use. Larvae were reared on diet which contained wheat-bran, sugar, brewer's yeast, Nipagin™, water and hydrochloric acid, while the adults were fed on sugar-yeast (3:1) and water (Tanaka et al., 1969). Third instar larvae (7 day old) were used for the pathogenicity screening tests. *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (mealworms) were cultured in vented plastic containers filled with fine bran, supplemented with carrots, while *G. mellonella* (wax moth) larvae were reared according the techniques used by Van Zyl and Malan (2015). Codling moth late-instar larvae, *Cydia pomonella* (Lepidoptera: Tortricidae), stored in larval diet covered by brown paper bags were obtained from Entomon Technologies (Pty) Ltd, and kept at 4°C until required.

Soil surveying

During early 2016 and 2017, sixty-eight soil samples were collected from a total of 18 orchards in Denau, Welgevallen, Baldric, Timberlea, Oak Valley and Fontana commercial fruit farms in the Western Cape Province and a further twelve from an organic farm, Carpe Diem, in the Northern Cape Province. These orchards had high fruit fly infestations which increased the probability of finding EPF, as it is expected that natural biological control agents are more likely to occur where fruit flies were present. Approximately 1 kg of soil was collected from each orchard and this was made up of a mixture of samples taken from across each orchard, following the method used by Malan et al. (2011). Collected soil was ideally moist and removed from about 5-10 cm depth, next to several fruit trees using an alcohol-cleaned hand spade. Soil samples were sieved directly into plastic containers and kept cool in the shade, before being transported to the laboratory.

Isolation and storage of fungi

To maximize EPF isolation, each soil sample was divided into four 250 g subsamples and processed separately. Distilled water was added to soil that became dry to keep it moist. Within five days of collecting the soil, each soil sub-sample was baited with five *Galleria* and mealworm larvae (Van Kleespies et al., 1989; Vanninen et al., 1989) and stored in a dark growth chamber at 25 ± 2°C. After five days, any dead or pupariating larvae were removed and replaced with an equivalent number of

live bait larvae. Each dead larva was put through a surface sterilization process to prevent the growth of bacteria or scavenger fungi. In this process, each larva was dipped in 70% alcohol, rinsed in distilled water, left for 1 min in bleach (NaOH), rinsed again in three different plates of distilled water and then placed on filter paper in a Petri dish (Lacey and Solter, 2012). Thereafter, 300 µl of distilled water was added to each Petri dish to provide moisture for potential fungal growth from the cadavers, all Petri dishes were sealed with Parafilm®, and stored at 25 ± 2°C in the dark. All soil samples were re-baited at least twice to ensure all EPF present in the soil were isolated (Malan et al., 2006; 2011).

In order to also isolate any Medfly-specific EPF present in the collected soil, the method used in Chapter 2 for EPN isolation was followed. In short, a 50 mm diam. Petri dish containing fruit fly diet was placed on top of 250 g of sieved soil from each site. The diet contained second and third instar *C. capitata* larvae, which were left to develop naturally and exit into the soil when ready to pupate, thus ensuring that the correct susceptible stage was entering the soil as an EPF bait. After one week, all dead Medfly larvae and pupae were sifted from the soil and processed using the sterilization process previously described. This technique ensured that all EPF, including Medfly-specific natural enemies, were isolated from the soil samples.

After 4-5 days, Petri dishes were checked and cadavers with visible signs of fungal growth (mycosis) were placed onto different Petri dishes containing Sabouraud Dextrose Agar (SDA; LAB, Neogen®) under a laminar flow hood before being sealed and stored in the dark at 25 ± 2°C. Those showing symptoms of a nematode infection were processed separately (see Chapter 2) and any with a foul smell or decomposed body were discarded. All SDA used throughout this study was prepared by placing 60 g of SDA (LAB, Neogen®) together with 1 L of distilled water in a 1 L glass bottle, which was shaken well before being autoclaved at 121°C for 21 min. Once cooled, agar was poured into small (50 mm diam.) and large (90 mm diam.) Petri dishes under a laminar flow hood.

To maintain clean cultures, conidia were collected from each fungal-infected larva and spread on a new SDA Petri dish. To ensure that the fungi isolated from the bait insects were indeed pathogenic to insects, Koch's postulates for pathogenicity were confirmed (Lacey and Solter, 2012; Steyn and Cloete, 1989). Conidial suspensions were prepared by collecting conidia from the sporulated SDA Petri dish and placing it in a 2 ml Eppendorf® tube containing 1ml distilled water and a drop of the wetting agent, Tween 80, because *Beauveria* and *Metarhizium* have hydrophobic cells (Wraight et al., 2007). Each tube was closed and vortexed for 2 min to produce a homogenous suspension (Goble et al., 2011). Five codling moth larvae or meal worms were dipped in the conidial suspension for 10 seconds and then placed on moist filter paper in a Petri dish (9 mm diameter). All Petri dishes were sealed with Parafilm® and kept in the dark at 25 ± 2°C. Insects were monitored for 14 days and only accepted as entomopathogenic if mycosis was observed (Figure 3.1).

Figure 3.1 Dead baited codling moth larvae showing mycosis caused by a *Metarizium* (left) and a *Beauveria* (right) EPF species, confirming that the isolated fungi from the soil were entomopathogenic.

EPF identification (extraction, amplification and sequencing)

Conidia from each confirmed EPF were added to multiple SDA plates and allowed to grow for 14-21 days in the dark at 25 ± 2°C. One fungal culture of each isolate was sent to Plant Protection Research Institute (PPRI, Pretoria, South Africa) for morphological identification and verification.

Molecular identification was carried out, using a separate fungal culture of each, in order to confirm the morphological identifications. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp.). For each fungal culture, about 200 mg of tissue (spores and hyphae) was scraped off the SDA plate, using a sterilized blade, and placed in a BashingBead™ lysis tube. The process was continued according to the protocol provided in the kit. To ensure the presence of DNA in the final product, 2 µL of each sample was tested using a Spectrophotometer ND-1000 (NanoDrop Technologies).

Two PCR primers were used to amplify the Internal Transcribed Spacer (ITS) rDNA regions, including ITS1, and the 5.8S and ITS2 ribosomal genes, as well as short parts of the 18S and 28S ribosomal genes. The primes TW81 5'-TTTCCGTAGGTGAACCTGC-3' (F) and AB28 5'-ATATGCTTAAGTTCAGCGGGT-3' (R) (Hominick et al., 2009) were used for sequencing with a cycle of 3 min at 95°C; followed by 35 cycles of 20 s at 95°C, 20 s at 48°C, and 30 s at 72°C; and a final cycle of 5 min at 72°C. The Central Analytical Facilities (CAF) at Stellenbosch University sequenced the PCR products. The forward and reverse sequences of each isolate were aligned and edited using CLC Main Workbench (ver. 7.9.1) and then blasted on Nucleotide BLAST® (NCBI, USA) to compare them against sequences from type material.

Pathogenicity of EPF

Source of EPF

EPF were obtained as formulated products from the respective manufacturers, as well as local soil surveys (Table 3.1). In order to standardize the EPF, each isolate was grown on SDA plates. The EPF isolated from the soil and those in the powder or oil formulation were suspended in 10 ml sterilized distilled water in a McCartney glass bottle and vortexed for 2 min. A 100 µl of each conidial suspension was spread on SDA using a sterile rod in a Petri dish (90 mm diam.), under a laminar flow hood, sealed with Parafilm® and placed in a dark growth chamber at 25 ± 2°C. Three plates were prepared for each EPF and allowed to grow for 21-25 days. New SDA plates and slants were prepared, using the conidia from the originally grown plates and, once fully grown and pure, they were stored in a fridge at 4°C until used.

Table 3.1 Name, species, isolate, formulation and source of EPF species (*Beauveria* and *Metarhizium*) tested against third instar *Ceratitis capitata* larvae in screening tests.

Name	Species	Isolate	Formulation	Source
6756	*B. bassiana*	PPRI6756	Spores	PPRI, Pretoria, South Africa
Broadband®	*B. bassiana*	PPRI5339	Oil	BASF Crop Protection, South Africa
EcoBb®	*B. bassiana*	R444	Powder	Plant Health Products, KwaZulu-Natal, South Africa
Meta69	*M. anisopliae*	ICIPE69	Oil	Real IPM, South Africa
MJ06	*M. robertsii*	MJ06	Spores	Soil sample from pear orchard, Baldric farm

Concentration

Most studies use a concentration between 1 x 10^6 conidia / ml and 1 x 10^8 conidia / ml when testing EPF pathogenicity against fruit flies. For the purpose of this study, a standard concentration of 1 x 10^7 conidia / ml was used for all EPF isolates. In order to have fresh conidia for the experiments, scrapings of each fungus were spread on an SDA plate and allowed to grow for approximately 2 weeks prior to experiments. Conidial suspensions were produced by pouring 10 ml of distilled water into the Petri dish containing the 2-3 week old fungal culture and using a glass slide to gently scrape loose conidia from the hyphae (de Lima Silva et al., 2016). The loosened mixture was poured into a McCartney glass bottle, through a square of organza material (Fabric Centre, Somerset West, South Africa) to filter out hyphae or agar pieces. Within 3 h, the concentration of conidia in the 10 ml

suspension was determined by placing 10 µl under either side of a cover slip on a Neubauer haemocytometer and diluting the stock according to the formula provided in Inglis et al. (2012).

Germination test

To ascertain the viability of the conidia, 100 µl of the 1 x 10^7 conidia / ml suspension was spread onto one SDA plate and, after being allowed to dry for 10 min, a sterile cover slip was placed on the surface. Each plate was sealed and placed in the dark at 25 ± 2°C and the percentage germination was examined after 18 – 24 hours from three random 100-spore counts from each plate (Ekesi et al., 2002). Propagules were considered viable if the length of the germ-tube was at least twice the diameter of that propagule (Inglis et al., 2012).

Screening of local and commercial EPF at different moisture levels

The pathogenicity of each EPF isolate to *C. capitata* third instar larvae was tested at a concentration of 1 x 10^7 conidia / ml per fruit fly larva, using the immersion or dipping method (Inglis et al., 2012). This method has previously been used to test EPF against various fruit fly species, including the Medfly (Beris et al., 2013; Quesada-Moraga et al., 2006; Toledo et al., 2006). The EPF isolates used for screening are indicated in Table 3.1. For each EPF, the pathogenicity screening was carried out in 12 alternate wells of a 24-well bioassay plate – a design often used when screening for the pathogenicity of entomopathogenic nematodes (e.g. Malan et al., 2011), but not previously used to test EPF pathogenicity. Filter paper (12.7 mm diam.) was added to each of the 12 wells. A drop of Tween 80 was added to the 10 ml conidial suspension and vortexed for 2 min. Twelve third instar larvae were then immersed in the suspension for approximately 20 sec before being placed separately into a well. Each bioassay plate was covered with a layer of tissue paper and a glass plate, to limit movement of jumping larvae between wells, and also secured with a rubber band. The entire process was repeated for each fungal isolate, and a control was prepared by dipping an equal number of larvae into a vortexed 10 ml of distilled water containing only Tween 80. Five replicate bioassay plates (n = 60) were inoculated in the same manner for each treatment and stored in 2 L plastic containers, lined with moist paper towels at 25 ± 2°C. The experiment was repeated on two separate dates, each time using a fresh batch of EPF.

The influence of moisture was investigated using all isolates, following the same procedure explained above, but without lining the 2 L containers with moist paper towels to create an experiment with a low moisture level.

For all the experiments, dead larvae or pupae with fungal growth were removed after the first 7 days, surface sterilized with 70% alcohol and placed on an SDA plate under a laminar flow hood. The remaining pupae were monitored daily for adult emergence and monitoring continued for 14 days from first adult emergence. As adults died, they were removed and sterilized in alcohol before being placed on SDA plates to facilitate mycosis. After the 14-day monitoring period, any un-emerged pupae were processed in the same manner. Mortality due to EPF was only accepted if fungal sporulation of the treated isolate was observed on the dead insects (Figure 3.2).

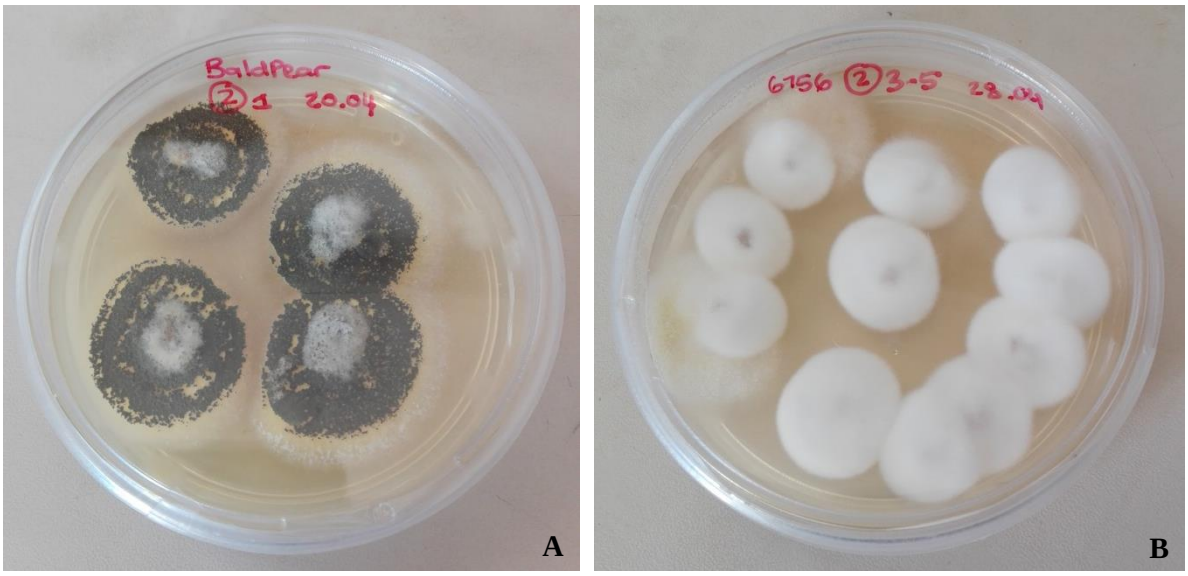

Figure 3.2 SDA plates with *Ceratitis capitata* larvae and pupae showing mycoses of a *Metarhizium* (A) and *Beauveria* (B) EPF isolate.

Sand bioassays

Sand bioassays were performed using two non-commercial EPF isolates, MJ06 and 6756. Distilled water (10 ml) was added to a 100 ml of sterilised Malmesbury river sand and placed in a 250 ml plastic container. Using a standard Potter spray tower (Burkard Scientific), a concentration of 1 x 10^7 conidia / ml (1 x 10^6 conidia / insect) was added to each container for each EPF treatment. This resulted in an area spray of approximately 1.05 x 10^5 conidia / cm^2 covering the sand surface. The Potter tower was also used to add 1 mL of only distilled water to the control treatment. Ethanol was sprayed in between treatments to sterilize the Potter tower and prevent any cross-contamination. Ten third instar *C. capitata* were added to each container and allowed to burrow into the sand naturally. This method simulates an application of EPF on the soil surface in the field, followed by Medfly larvae exiting fruit to pupate beneath the soil. There were 10 replicates of each treatment (n = 100 fruit fly larvae) and all containers were covered and stored in the dark at 25 ± 2°C for 14 days. After two weeks, all dead larvae, pupae and emerged adult flies were surface sterilized in 70% alcohol and placed on SDA plates (Goble et al., 2011). Mortality due to EPF was determined by recording failure

of larvae or pupae to emerge and death of adults, together with consequent mycosis of each dead insect.

Statistical analyses

The two batches from the screening experiment were compared for statistical differences using a t-test. The batches were pooled and analysed using a one-way analysis of variance (ANOVA) to compare the differences in efficacy of the EPF isolates. Differences between isolates were determined by the Least Significant Difference post-hoc test. The follow-up experiment, which excluded moisture, was similarly analysed with a one-way ANOVA and subsequent Bonferroni post-hoc test. For all ANOVAs performed, a Shapiro-Wilk test was used to assess normality of the residuals and a Levene's test for homogeneity of variance. Where data were found to be non-normal, Kruskall-Wallis tests and subsequent multiple comparison tests were used to confirm ANOVA results. In the sand bioassay, the virulence of the two EPF was assessed using a one-way ANOVA followed by a Games-Howell post-hoc test since the Levene's test showed significant non-homogeneity among the variances. All analyses were performed using STATISTICA 13.0 (StatSoft Inc., 2016).

Results

Soil surveying

A total of 21 EPF isolates were collected, using three different bait insect species (Figure 3.3), from soil collected from nine different orchards, on four different farms. EPF which were identified are presented in Table 3.2. Majority of the species identified morphologically belonged to the *Metarhizium anisopliae* (Metschnikoff) (Sorokin) complex, and based on molecular sequencing, which showed 99% identity and query coverage with type material, they are likely the local species *Metarhizium robertsii* (Bisch, Rehner and Humber), which is found within this complex. Two of the isolates were morphologically and using molecular tools identified as *Beauveria bassiana* (Balsamo) (Vuillemin). A nematophagous fungus was isolated from a soil sample taken from a commercial pear orchard and morphologically identified as *Purpureocillium lilacinum* (Thom) (Luangsa-ard, Houbraken, Hywel-Jones and Samson). Importantly, one of the isolates belonging to the *M. anisopliae* complex, MJ06, was isolated directly from a *C. capitata* larva.

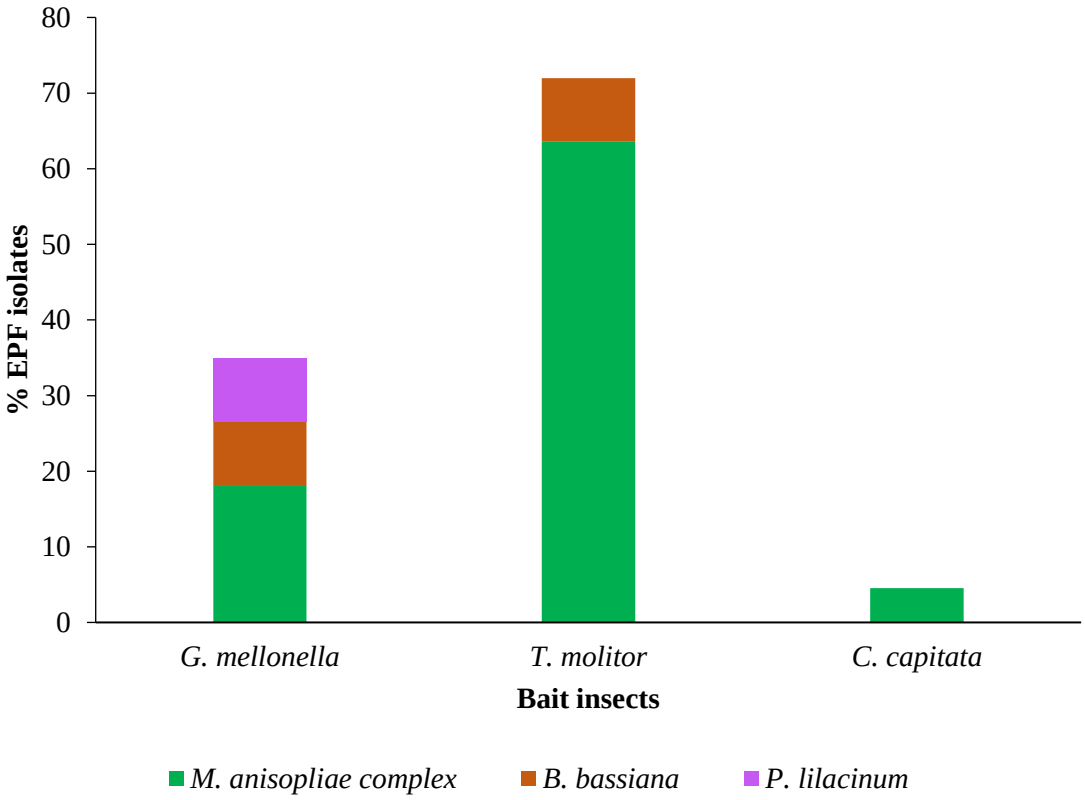

Figure 3.3 Percentage of entomopathogenic fungi (EPF) isolates obtained from local soil samples by trapping with three different bait insect species, including larvae of the target pest, *Ceratitis capitata*. Different colours represent isolates belonging to species of *Metarhizium*, *Beauveria* and *Purpureocillium*.

Table 3.2 Details of the local entomopathogenic fungi isolated and identified from soil samples taken in the Western Cape Province and in Upington (Northern Cape Province). Morphological identifications by Dr M. Truter.

Morphological ID	Molecular ID	Isolate	Host species	Fruit	Farm	Area	GPS	PPRI No.
Metarhizium anisopliae complex	*Metarhizium robertsii*	MJ06	*Ceratitis capitata*	Pear	Baldric	Stellenbosch	S33° 55.664' E18° 57.422'	23949
M. anisopliae complex	*M. robertsii*	MJ80	*Galleria mellonella*	Apple	Oak Valley	Grabouw	S34° 08.773' E19° 03.573'	23950
M. anisopliae complex	-	MJ20	*Tenebrio molitor*	Pear	Timberlea	Stellenbosch	S33° 54.201' E18° 51.681'	23951
M. anisopliae complex	*M. robertsii*	MJ48	*T. molitor*	Apple	Baldric	Stellenbosch	S34° 08.817' E19° 03.516'	23953
M. anisopliae complex	*M. robertsii*	MJ17	*T. molitor*	Pear	Timberlea	Stellenbosch	S33° 54.201' E18° 51.681'	23954
M. anisopliae complex	*M. robertsii*	MJ69	*T. molitor*	Apple	Oak Valley	Grabouw	S34° 08.773' E19° 03.573'	23955
M. anisopliae complex	*M. robertsii*	MJ96	*G. mellonella*	Pear	Oak Valley	Grabouw	S34° 08.886' E19° 03.509'	23956
M. anisopliae complex	*M. robertsii*	MJ52	*T. molitor*	Pear	Baldric	Stellenbosch	S33° 55.664' E18° 57.422'	23958
M. anisopliae complex	*M. robertsii*	MJ39	*T. molitor*	Plum	Baldric	Stellenbosch	S33° 55.663' E18° 57.328'	23959
Purpureocillium lilacinum	-	MJ96b	*G. mellonella*	Pear	Oak Valley	Grabouw	S34° 08.886' E19° 03.509'	23957
Beauveria bassiana	*B. bassiana*	MJ24	*T. molitor*	Peach	Timberlea	Stellenbosch	S33° 54.195' E18° 51.750'	23952
Beauveria bassiana	*B. bassiana*	Upington1A	*G. mellonella*	Raisins	Carpe Diem	Upington	S28° 27.012' E21° 19.000'	24198

Screening of commercial and local EPF

In the viability tests, the average germination of conidia for all EPF tested ranged between 84.67% and 100%. When third instar larvae were immersed in a conidial suspension of 1 x 10^7 conidia / ml, all EPF were found to be pathogenic against *C. capitata*. There was no significant difference between the batches and the data were pooled (t = 0.762614, P = 0.449). The distribution of the combined data of the repeats was close to normal (W = 0.95911, P = 0.043) and the assumption of homogeneity of variance was met ($F_{(5, 54)}$ = 0.952585, P = 0.455). The one-way ANOVA showed that at least one of the treatments differed significantly from the rest (Figure 3.4; $F_{(5, 54)}$ = 9.7401, P = 0.00). The post-hoc test revealed that all EPF differed significantly from the control and that MJ06 caused significantly higher percentage mycosis than Broadband® (P = 0.046). Mortality of *C. capitata* caused by both local soil isolates and commercial EPF products at a concentration of 1 x 10^7 conidial / ml ranged between 56.67 – 74.17%. When considering the life stage at which the treated *C. capitata* larvae died and mycosed, it was found that mycosis occurred at all four stages (larva, pupa, emerging adult, and adult) for most of the treatments (Table 3.3; Figure 3.5 and 3.6). The majority died as adults, but many were also killed as pupae, specifically by isolates MJ06 and 6756. Overall, very few larvae and emerging flies' mycosed. No mycosis was found in any of the controls and natural deaths in the control were mainly larvae.

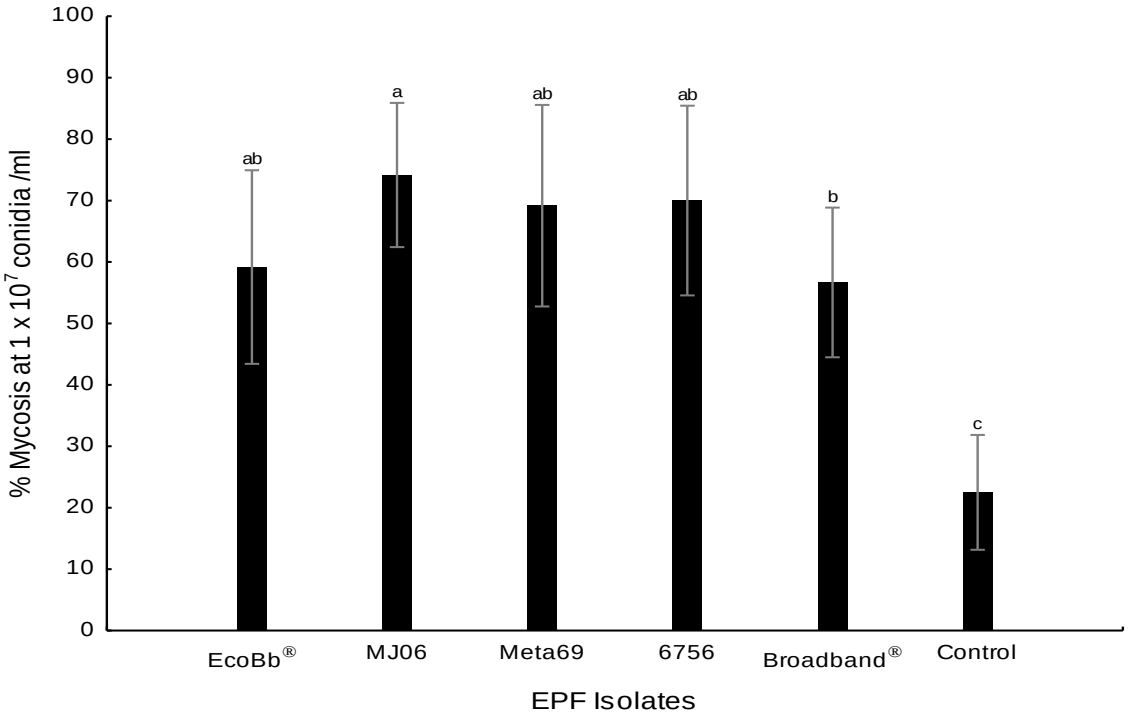

Figure 3.4 Mean percentage mycosis (95% confidence interval) of third instar *Ceratitis capitata* after being immersed in fungal suspensions of five different EPF at a concentration of 1 x 10^7 conidia / ml. Mortality data (natural deaths) of the water-only control are presented. Different letters above bars indicate significant differences.

Table 3.3 Percentage of different life stages of *Ceratitis capitata* which mycosed in each of the two experiments, after third instar larvae were immersed in 5ml of inoculum at a concentration of 1 x 10^7 conidia / ml. Mortality data are presented for the water-only control.

Moisture?	% Mycosed	EPF Isolates					
		EcoBb®	MJ06	Meta69	6756	Broadband®	Control
Yes	Larvae	9.2	5.0	13.3	7.5	10	13.3
	Pupae	4.2	22.5	10	26.7	4.2	7.5
	Emerging adults	0.0	0.8	1.7	0.0	2.5	0.0
	Adults	45.8	45.8	44.2	35.8	40	1.7
	Stages not mycosed*	40.8	25.9	30.8	30.0	43.3	77.5
No	Larvae	0.0	0.0	0.0	0.0	0.0	14.2
	Pupae	6.7	30	20	5	6.7	2.5
	Emerging adults	0.0	0.0	0.0	0.0	0.0	0.0
	Adults	21.7	25	3.3	5	31.7	0.0
	Stages not mycosed*	71.6	45	76.7	90	61.6	83.3

*this is the sum of live flies after the final evaluation and those showing no mycosis.

In the viability tests of the experiment excluding moisture, the average germination of conidia for all EPF tested ranged between 86.33% and 100%. A one-way ANOVA of the data indicates differences between the treatments (Figure 3.7; $F_{(5, 29)} = 5.4692$, $P = 0.001$). The post-hoc test revealed significant differences between the percentage mycosis of isolates MJ06 and 6756 ($P = 0.002$) and MJ06 differed significantly from the percentage mortality in the control ($P = 0.002$). In treatments without moisture, no larvae or emerging adults had mycosed (Table 3.3). Isolate MJ06 caused high mycosis for both pupae and adults, while very few were affected by isolate 6756. Meta69 mainly caused pupae to mycose, while EcoBb® and Broadband® mostly affected the adult Medflies.

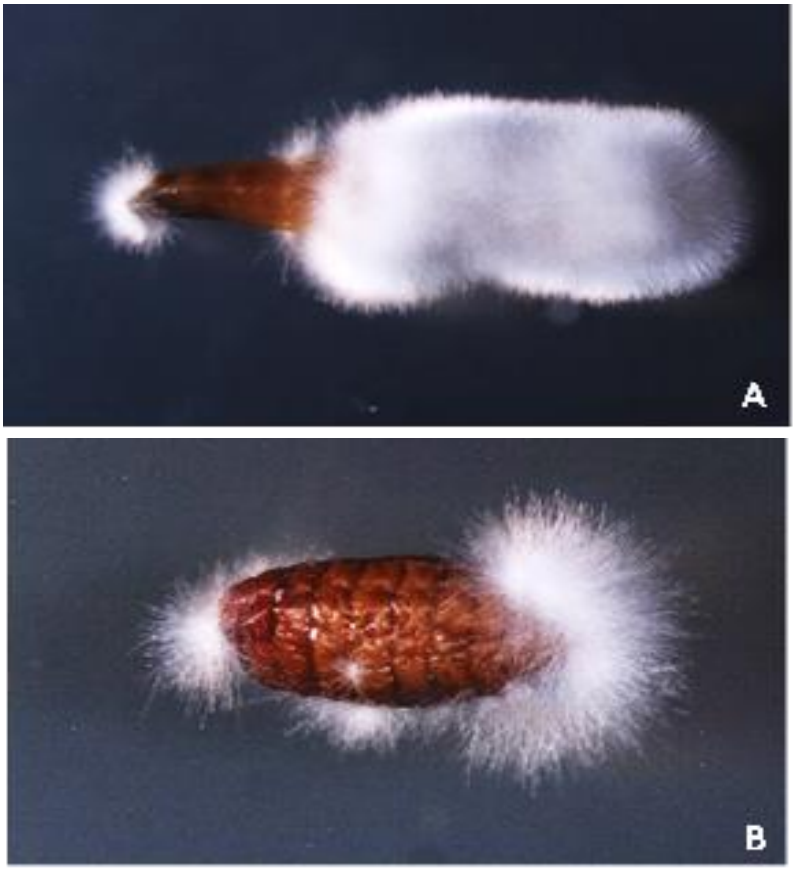

Figure 3.5 Mycosed *Ceratitis capitata* larva (A) and pupa (B) which died after being immersed in 5 ml suspension of Broadband® and Meta69, respectively, at a concentration of 1 x 10^7 conidia / ml, and maintained at 25 ± 2°C in a moist 2 L chamber.

Figure 3.6 Mycosed *Ceratitis capitata* adults which died after being immersed in 5 ml suspension of *Beauveria bassiana* (6756) (A) and EcoBb® (B), at a concentration of 1 x 10^7 conidia / ml, and maintained at 25 ± 2°C in a moist 2 L.

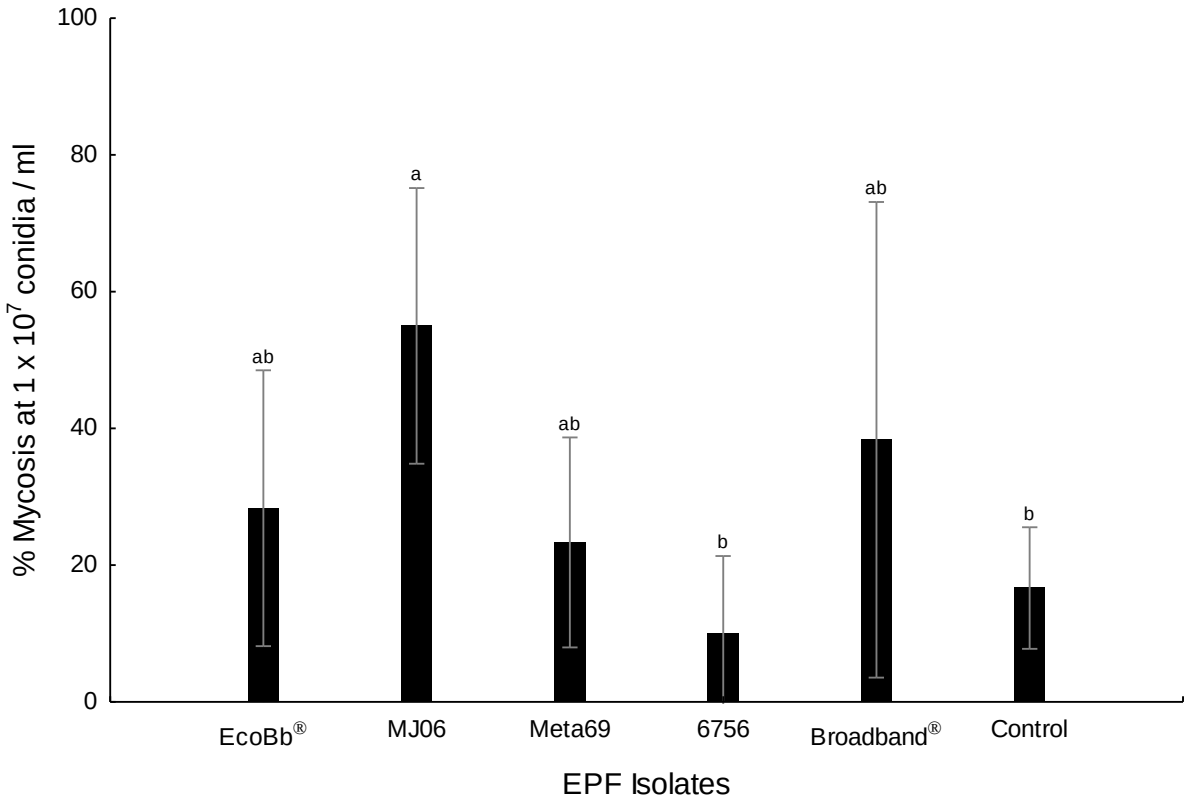

Figure 3.7 Mean percentage mycosis (95% confidence interval) of third instar *Ceratitis capitata* after being immersed in fungal suspensions (5 ml) of five different EPF at a concentration of 1 x 10^7 conidia / ml, and placed at 25 ± 2°C with no moisture. Mortality data (natural deaths) of the water-only control are presented. Different letters above bars indicate significant differences.

Sand bioassays

In the viability tests, germination of conidia for isolate MJ06 ranged from 97% to 100%, and for isolate 6756 it was between 94% and 100%. Analysis of the sand bioassay data revealed that both EPF tested differ significantly from the control ($F_{(2.27)} = 31.612$, $P = 0.00$), but not from each other (Figure 3.8). In this more natural condition, isolate 6756 caused an average of 86% mycosis, while MJ06 caused an average of 62%. For both EPF tested, most of the exposed larvae died as adults (Table 3.4). A very small percentage died as third instar larvae or pupae, and none died while emerging as adults from the sand.

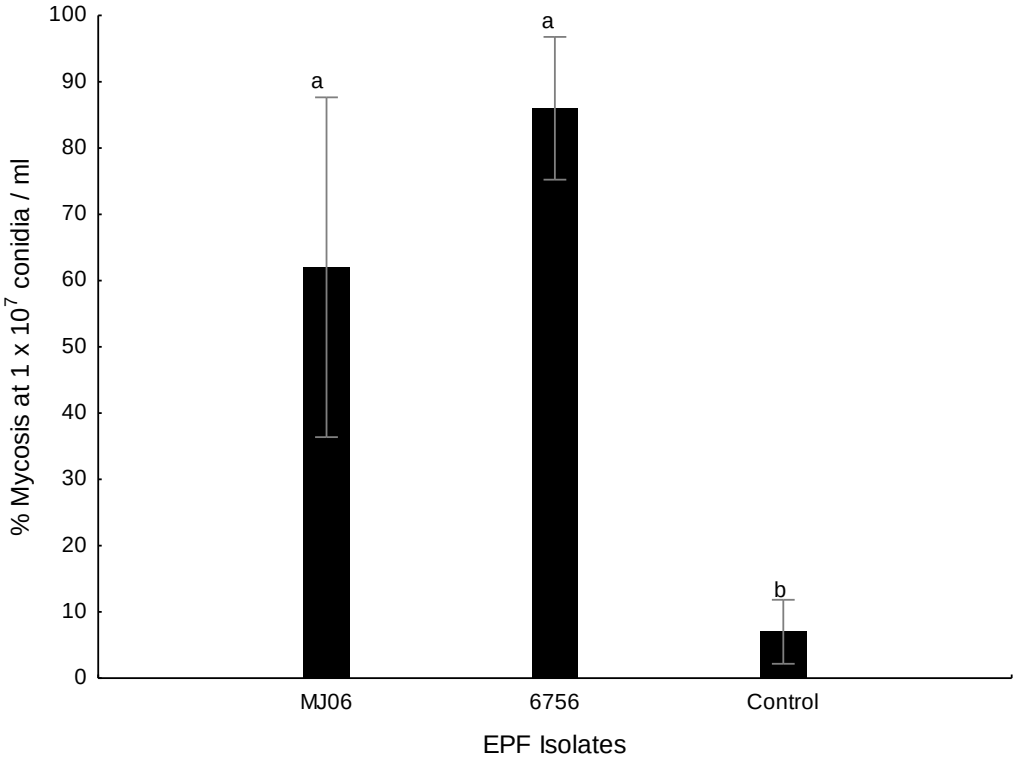

Figure 3.8 Mean percentage mycosis (95% confidence interval) of third instar *Ceratitis capitata* larvae that were exposed in sand to *Beauveria bassiana* (6756) and MJ06 at a concentration of 1 x 10^7 conidia / ml (of 1 x 10^6 conidia / insect), as well as a water-only control. Data displayed for the control represents percentage mortality of *C. capitata*. Different letters on top of the bars indicate significant differences.

Table 3.4 Number of different life stages of *Ceratitis capitata* showing mycosis after being exposed as third instar larvae to 1 x 10^6 conidia /insect in 100 ml of sand for 14 days. Mortality data are presented for the water-only control.

% Mycosed	**EPF Isolates**		
	MJ06	6756	Control
Larvae	1.0	2.0	0.0
Pupae	4.0	2.0	6.0
Emerging adults	0.0	0.0	0.0
Adults	57	82	1.0
Stages not mycosed*	38	14	93
Total	100	100	100

*this is the sum of live flies after the final evaluation and those showing no mycosis.

Discussion

The efficacy or virulence of an entomopathogenic fungus can vary between isolates and against different hosts (Lacey et al., 2015). Thus, the continuous exploration for EPF in the form of soil sampling, especially in areas of intended fruit fly control, is important. Baiting soil samples with larvae of the highly susceptible *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) (wax moth) has proven to be a simple and successful method for isolation of such entomopathogens (Goble et al., 2010; Hatting et al., 2004; Zimmerman, 1986). Goble et al. (2010) isolated EPF from soil samples by baiting with susceptible wax moth larvae, as well as the target pests, Medfly and false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), both of which were also able to isolate EPF, but not as abundantly as the wax moth larvae. In this study, four different fruit farms yielded more than 20 EPF isolates, suggesting that natural control agents are abundant in the soil. Most of these belong to the *Metarhizium anisopliae* complex, which includes the closely-related species *Metarhizium pingshaense* (Chen and Guo), *Metarhizium anisopliae* (Metschn.) (Sorokin), *Metarhizium robertsii* (Bisch, Rehner and Humber) and *Metarhizium brunneum* (Petch) (Bischoff et al., 2009). However, using morphological identification, which involves the observation of the asexual characters including conidia and conidiogenous cells (Inglis et al., 2001), was not sufficient in distinguishing between these species. When sequences of the ITS rDNA regions were amplified and analysed for molecular identification, all isolates within this complex were most closely related to the type material of *M. robertsii*. However, further sequencing of the elongation 1-alpha (EF 1-α) and β-tubulin gene would enable more accurate species identification (Bischoff et al., 2009; D'Alessandro et al., 2014; Abaajeh and Nchu, 2015). In this study, *M. robertsii* was found to be the most dominant species in the soil sampled. It was also the most common species in a recent study by Abeejah and Nchu (2015), which was carried out within areas of the Western Cape Province, where the species presence was first reported in South Africa. There is no literature indicating the screening of this strain against fruit flies, but previous studies may have tested *M. robertsii* as a *M. anisopliae* strain - not yet reclassified within the complex (Bischoff et al., 2009).

When isolating EPF, Sookar et al. (2008) found that temperature influenced isolation, with *B. bassiana* more frequently isolated at 15°C. The current study used a constant temperature of 25°C, which may have favoured the isolation of *Metarizium* species (Sookar et al., 2008). However, the study by Goble et al. (2010) yielded more isolates of *B. bassiana* than *M. anisolpliae* var. *anisopliae* and their soil samples were incubated at 22°C, suggesting that temperature is not the only factor influencing these isolations. In the current study, two strains of *B. bassiana* were isolated from farms in Stellenbosch and Upington. This species has been known to be an important biological control agent since it was first described nearly 180 years ago (Zimmerman, 2007). In South Africa, isolates of *B. bassiana* have been used to formulate two commercial products, EcoBb® and Broadband®,

which are registered for the control of whiteflies, red spider mites, thrips, red scale and several moth species (BASF Crop Protection, 2017; Plant Health Products (Pty) Ltd, 2017).

Only one isolate of *Purpureocillium lilacinum* (Thom) (Luangsa-ard, Houbraken, Hywel-Jones and Samson), formerly known as *Paecilomyces lilacinus* (Thom) (Samson), was isolated in this study. In a recent study, Abaajeh and Nchu (2015) obtained eight isolates of this species from soils collected from various "disturbed sites" in the Western Cape. This species is used to control plant-parasitic nematodes (Mitchell et al., 1987), but has also been found to cause infections in immuno-compromised patients. The inability to distinguish which isolates of this species causes infection makes the use of this species in pest management a health risk to humans (Luangsa-ard et al., 2011).

Entomopathogenic fungi are able to invade their hosts by penetration of the cuticle, thereby providing them with an advantage over other insect pathogens for use in pest control. However, there needs to be compatibility between the EPF and its host in order for infection to occur (Castrillo et al., 2005). In this study, all five EPF tested were pathogenic against *C. capitata* at a concentration of 1 x 10^7 conidia / ml. This was expected for Meta69, which has previously been screened against Medfly (Ekesi et al., 2002), but is an important finding for *Beauveria bassiana* (6756) and the *B. bassiana*-based commercial products. Screening of commercial products against different pests, not registered on the label, is important, as increased knowledge on the ability of the product to control multiple pests will enhance the use of these more environmentally-friendly control methods (Wraight et al., 2007). Furthermore, it could lead to extension of the product label, thus registering for control against a wider range of pests. Of the commercial products, Broadband® was the only product to cause significantly lower percentage mycosis than the local isolate MJ06, although it did not differ in performance to the other two commercial products. Broadband® is not registered against fruit flies, but offered consistent control of *C. capitata* in this study, and has also shown good control against the false codling moth (Goble et al., 2011). Meta69, which is a *M. anisopliae* isolate (ICIPE 69), was previously tested against third instar larvae of the Medfly, by exposing the insects to sand thoroughly mixed with the conidial suspension (Ekesi et al., 2002). In their study, they found 90% of the larvae to mycose as pupae. This is not directly comparable to the results in our study as they used a different experimental design, including a higher concentration (1 x 10^8 conidia / ml), but it is surprising that so few pupae mycosed in our initial experiments. In a follow-up experiment using ICIPE69, Ekesi et al. (2003) found that this isolate does not perform as well in "wetter soils". This could explain the relatively high percentage of mycosed pupae caused by Meta69 in our experiment without moisture.

Abiotic environmental factors, specifically moisture, temperature and solar radiation, have a strong effect on fungal survival, but also on their efficacy as a control (Zimmermann, 2007). Some insight on the influence of moisture was provided in this study, although further testing in sand bioassays and field trials are required. Suboptimal conditions such as low moisture or low temperature may result in

the 'resting' of fungal structures within the dead host and could fail to visually mycose (Pendland, 1982; Castrillo et al., 2005). The influence of moisture on the success of the agent may also depend on the isolate of EPF or insect host. For example, increased soil moisture level had no effect on the number of mycosed *Rhagoletis indifferens* (Curran) (Diptera: Tephritidae) larvae exposed to two *B. bassiana* isolates (Cossentine et al., 2010). Furthermore, the present study only utilized a set temperature for all experiments (25°C). This factor may have been responsible for some of the differences observed, especially in the *B. bassiana* isolates, which in soil surveys have been found to cause greater infection at lower temperatures (15°C) (Sookar et al., 2008).

Local species have the added advantage of being adapted to local climatic conditions. This study found that the local soil-sampled MJ06, which was baited with the target host *C. capitata*, was able to provide a high level of control, irrespective of the amount of moisture in the treatment. This suggests that this isolate may remain virulent against *C. capitata* even when conditions are dry and slightly unfavourable for the EPF. This may be an important consideration in the context of the Western Cape, which experiences hot and dry summers. The ability of the commercial products to show virulence without moisture against *C. capitata*, although rather low, is further a positive finding in the context of the Western Cape. However, the experiment related to low moisture needs to be repeated to verify results and account for biological differences (Bell, 2016). MJ06 also caused high mortality of pupae, with fewer adults emerging, highlighting its ability to offer effective control against the soil life stages of this pest. The other local isolate tested, 6756, only caused a high level of mycosis when moisture was incorporated into the experiment. Moisture may thus influence its ability to cause greater infectivity of Medflies. In addition, most larvae exposed to 6756 died and mycosed as pupae or adults. Thus, this EPF may have penetrated and killed the target insect, but moisture was required for germination and growth of the fungi (Lacey et al., 2015). Therefore, because fungal isolates may vary in their response to environmental conditions due to being "independent biological entities" (Garrido-Jurado et al., 2011), selection of EPF should consider isolates that can withstand the conditions of the agro-ecology wherein they will be applied.

When larvae were exposed to sand inoculated with the two non-commercial isolates, MJ06 and 6756, there was high adult mycosis. Previous studies have found significant mortality to occur on adult fruit flies following exposure of larvae or pupae to EPF (Castillo et al., 2000; Lezama-Gutierrez et al., 2000; Ekesi et al., 2002; Goble et al., 2011). Although this experiment sought to assess control of the soil life stages, the high infectivity of adults was demonstrated. As alluded to earlier, the time when infection occurs to the *C. capitata* soil stage is unclear – and in these bioassays it is even more likely that infection occurred during or after emergence, rather than as larvae. Other studies screening pathogenicity of EPF, generally exposed third instar fruit fly larvae to a substrate (e.g. soil, sand, vermiculite) which had been inoculated with the conidial suspension mixed thoroughly into the substrate (Ekesi et al., 2002; Cossentine et al., 2010; Goble et al., 2011). In this study, the sand

bioassay did not serve as a screening experiment, but instead assessed the efficacy of EPF in a manner which more closely resembled its' functioning in the field. Mycosis of adults showed that not only can soil treatments with MJ06 and 6756 assist in fruit fly control, but also highlights the potential of using these two non-commercial isolates for control of the adults. Further studies could test these products for use in autoinoculation devices, where adults are attracted, infected and killed by the EPF (Dimbi et al., 2003; 2013) or in bait sprays (Beris et al., 2013). Infectivity of adults also offers the potential of horizontal transmission of the fungal conidia during mating or mating attempts (Quesada-Moraga et al., 2008) and thus may enhance the spread of the fungi. This is because adults may still be able to fly a long distance before succumbing to the EPF and subsequently becoming a new source of infection to other flies (Goble et al., 2011).

To our knowledge, this is the first time bioassay plates were used to screen pathogenicity and virulence of EPF against fruit flies. This method provides a simple way of assessing which strains are most virulent, and can be used to identify which isolates should be selected for further research. The design of the initial experiments ensured that no horizontal transmission would occur. This was problematic in previous studies (Ekesi et al., 2002; Goble et al., 2011), and the use of bioassay plates is therefore recommended for screening pathogenicity of various EPF isolates. However, the emerged adults were confined to the well and may have come into direct contact with conidia after emerging, rather than being infected as larvae and only dying as adults. Identifying the exact time of infection therefore becomes a challenge in deciding the optimal soil stage to target. Trials ran for at least 14 days and thus all flies would have died during this time. This is due to the impracticality of providing food, which is a trade-off in the experiments design, and this may have stressed the adults near the end of the experiment, leading to potential increased susceptibility to fungal infection (Inglis et al., 2001). However, it is unlikely that any infections happened after death as the initiation of fungal infection has been found to be related to host cues (physical and/or chemical) and the insects must have therefore been alive when attacked by the EPF (Castrillo et al., 2005). Overall, analysis of mycosis, rather than only mortality, for all life stages is a necessary step to confirm EPF infection (Ekesi et al., 2002; 2003).

This study identified local EPF isolates belonging to three different genera, isolated from soils collected from commercial farms in the Western and Northern Cape Provinces of South Africa. The majority were closely related to the species *M. robertsii*. The isolation of a *Metarhizium* species (isolate MJ06) using a *C. capitata* larvae highlighted the importance of baiting soil samples with larvae of the intended host. This isolation provided an EPF strain competitively virulent against the target pest and, therefore, a potential biological control agent. Isolate MJ06 was able to infect *C. capitata*, regardless of the level of humidity, causing significant fungal mycosis of both pupae and adults. Similarly, the non-commercial isolate of *Beauveria bassiana* (6756), showed promise, causing high mortality and mycosis in the screening and sand bioassays. These isolates should be considered

in future research studies in order to assess their performance in field conditions. The pathogenicity of the commercially available EPF products, EcoBb®, Broadband® and Meta69, against *C. capitata* observed in this study should provide encouragement for the increased use of such alternatives for the control of agricultural pests, including the Mediterranean fruit fly.

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CHAPTER 4

Surveying for parasitoid wasps (Hymenoptera: Parasitica) associated with fruit-infesting fruit flies across three South African Provinces

Introduction

Fruit flies (Diptera: Tephritidae) are economically important pests in fruit-growing areas across the world. They cause extensive economic losses due to their direct damage by larval feeding, require expensive controls and also pose restrictions to the export market (White and Elson-Harris, 1992). Among the known fruit flies, the polyphagous Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), or Medfly, is an important pest species to fruit growers globally, including those in South Africa (Manrakhan and Addison, 2014). It belongs to a genus which is thought to have a sub-Saharan origin, but it has become widespread, attacking nearly 400 host plants, and has the ability to produce several generations within a year (De Meyer, 2000; De Meyer et al., 2002). In South Africa, most tephritid pests attacking commercial fruits are from the genera *Ceratitis* (MacLeay) and *Dacus* (Fabricius), with *C. capitata*, *C. rosa* (Karsh), *Ceratitis quilicii* (De Meyer, Mwatawala and Virgilio), and *C. cosyra* (Walker) being among the most important fruit fly pests, all of which are quarantine pests highlighted in international quarantine restrictions (Barnes et al., 2002; De Meyer et al., 2016; Grové and De Beer, 2014). A recent invader, *Bactrocera dorsalis* (Drew, Tsuruta and White), is further threatening the fruit industry in the country (Grové and De Beer, 2014).

Current fruit fly control methods mainly aim to control the adults using insecticidal sprays, bait applications, and release of sterile males whereby mating with wild females results in infertile eggs (Barnes et al., 2015). Orchard sanitation, which is an important practice aiming to remove dropped fruits infested with larvae, and thus prevent the formation of pupae and adults, is highly recommended but this is often neglected due to the high labour costs. Among the available control options, chemical pesticides dominate as the main control strategy (Peck and McQuate, 2000), but their known negative effects on the environment, human health, non-target organisms and the potential of resistance build-up is a driving force behind the need for more sustainable alternatives (Ekesi et al., 2002).

The use of natural enemies as biological control agents is an important alternative in insect pest management. Parasitic wasps (Hymenoptera: Parasitica) or parasitoids are wasps that use insects as hosts to lay their eggs, allowing their larvae to feed and live as parasites, eventually emerging as adults and killing the host (Karagoz et al., 2009). Many are able to parasitize fruit-infesting tephritids, often attacking them when they are hidden within the fruit as eggs and larvae, or in the soil as pupae (Wharton et al., 2000). Successful use of African parasitoids to control *C. capitata* in Hawaii in 1913 has led to the global focus on the use of these natural enemies as a sustainable control strategy within

an overall IPM strategy (Jenkins et al., 2012; Silvestri, 1913). Parasitoids can be effective control agents as they are target specific, mobile, pre-adapted to searching for their cryptic host, and rarely pose a threat to other organisms (Headrick and Goeden, 1996). For example, in Hawaii the egg-pupal parasitoid, *Fopius arisanus* (Sonan), was able to reduce *B. dorsalis* populations by up to 70% by attacking eggs within ripe fruits (Vargas et al., 2012). However, the importation of exotic parasitoids for the purpose of biological control may pose an inadvertent risk to non-target organisms and may result in competition with existing native enemies (Bokonon-Ganta et al., 2007). It is thus important to identify and investigate the use of locally occurring parasitoids for fruit fly control prior to investing in a classical biological control programme.

Exploration for fruit fly parasitoid wasps has mainly involved the collection of fruit infested by fruit flies and rearing out flies and parasitoids under optimized conditions (Copeland et al., 2006; Garcia and Corseuil, 2004; Manrakhan et al., 2010; Wharton et al., 2000). This is a simple surveying method, which can easily be carried out on a large scale using various host fruits. For instance, a recent study in Ghana studying the parasitoids associated with tephritids, recovered four braconid wasps, that were reared from 17 host fruit species infested with fruit flies (Badii et al., 2016). Although this method will provide useful information on the parasitoids associated with certain fruit fly species, identification of specific host-enemy relationships often remains unknown, unless early life stages are laboriously removed from the collected fruit (Jenkins et al., 2012; Wharton et al., 2000). Furthermore, fruit collection does not allow for the exploration of pupal-attacking parasitoids (Manrakhan et al., 2010).

In order to identify Hymenoptera specifically attacking *C. capitata*, an improved surveying method employing the use of sentinel traps has been successfully tried in various insect studies. This method refers to setting the target host in the field as bait for the parasitoids, and then rearing out the parasitoids in the laboratory. Hymenopteran parasitoids were recovered from lab-reared stinkbug eggs which were placed in vegetable crop fields (Koppel et al., 2009); from eggs and larvae of the South American tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) (Abbes et al., 2014); and native parasitoids of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) were obtained using sentinel eggs, larvae and pupae (Gabarra et al., 2015; Rossi Stacconi et al., 2013). A recent study in Tunisia made use of Medfly infested fruit and exposed Medfly pupae to successfully identify a native Medfly parasitoid, which could potentially be used as a biocontrol agent (Harbi et al., 2015). Other work on fruit flies using sentinel trapping mostly aimed to assess parasitism rates of known parasitoids (Eitam and Vargas, 2007; Ero, 2009).

In order to utilise the recovered parasitoids as potential biocontrol agents, they also need to be correctly identified (Jenkins et al., 2012). Morphological identification is the traditional method and involves the use of keys, which are created based on various identifying characteristics. Numerous

books and keys have been developed for the Hymenoptera (Goulet and Huber, 1993), but there is insufficient information on wasps - specifically those associated with tephritid fruit flies. One available online resource, Parasitoids of Fruit-Infesting Tephritidae or Paroffit (www.paroffit.org), provides keys on fruit fly parasitoids, but most information is focussed on parasitoids of the olive fruit fly, *Bactrocera oleae* (Rossi). Furthermore, the size and intricacies of fruit fly parasitoids can make morphological identifications difficult and are often only successful with the help of taxonomic experts, who are increasingly becoming fewer.

Molecular techniques have become increasingly popular for insect identifications (Gariepy et al., 2007; Zaldivar-Riveron et al., 2006). DNA barcoding is the most commonly used technique and often involves PCR amplification and sequencing of the mitochondrial cytochrome oxidase I gene (COI). Several global studies on tephritid fruit flies and their parasitoids have utilised COI barcoding (Quicke et al., 2012; Rugman-Jones et al., 2009; Santos et al., 2011). An important benefit is that such techniques can be employed by non-experts and do not require specialised knowledge (Jenkins et al., 2012). A drawback of barcoding is that species can only be accurately identified if matched to comparable sequences within a large database (Jenkins et al., 2012).

In South Africa, there is limited information on the species of fruit fly parasitoids present as very few surveys have been conducted. Previous studies, however, have explored the parasitoids of the olive fruit fly, *B. oleae* (Mkize et al., 2008; Neuenschwander, 1982) and parasitoids associated with fruit flies reared from coffee berries in Mpumalanga (Manrakhan et al., 2010). These studies found just over 30 parasitoid species associated with fruit flies across several South African Provinces. However, additional information on parasitoids reared from other host fruit, as well as other potential surveying methods for parasitoids in different regions of South Africa is crucial to inform biological control efforts in the country. Furthermore, the parasitoid complex of new invasive species, such as *B. dorsalis,* and recently described species, such as *C. quilicii,* is not well understood. Therefore, the aim of this study was to collect, using different sampling methods, and identify parasitoid wasps associated with fruit flies in the Western Cape, Mpumalanga and Limpopo Provinces of South Africa. Sampling was focussed only in the Western Cape, while the Mpumalanga and Limpopo samples were obtained from previously collected fruit-rearing samplings, but which had never been identified.

Materials and Methods

Collection of parasitoid wasps

Collection of fruit fly infested fruit

Commercial fruits were sampled at several farms with known fruit fly problems in the Western Cape Province between January and March 2016 (Figure 4.1). Only visibly damaged fruit with clear

oviposition holes or small brown spots were sampled to maximise the chance of finding fruit flies as well as their parasitoids. One orchard block per fruit type was selected and a minimum of 50 ground and 50 tree fruits were randomly collected and held separately. It was important to sample at both levels as different parasitoid species may prefer or avoid fruit from either the ground or those still on the tree (Eitam and Vargas, 2007). Sampled fruit were placed in clear plastic or brown paper bags, clearly labelled and placed in the shade. All fruit were transported in a large cooler box to the Insectary at Stellenbosch University within two hours for processing.

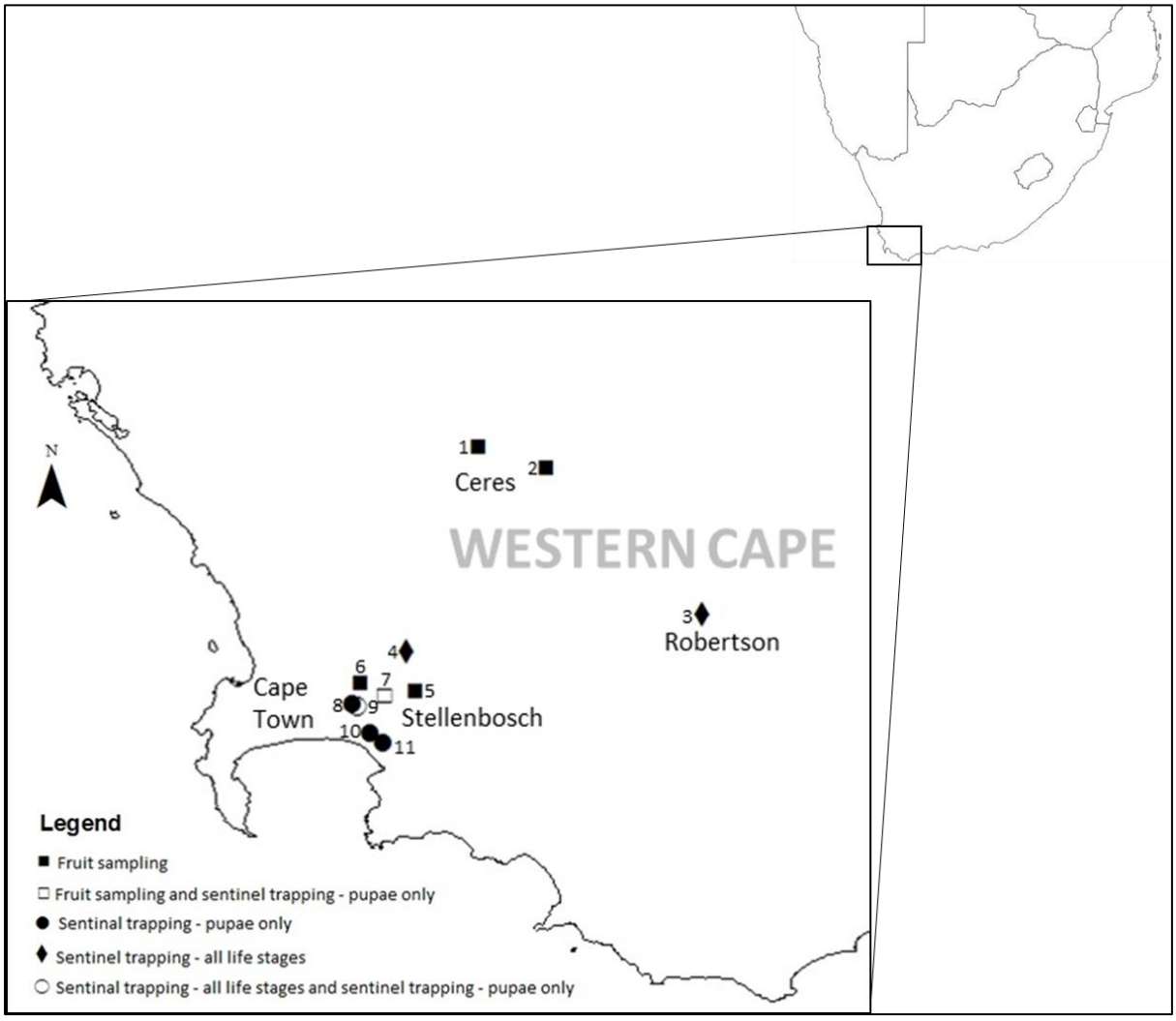

Figure 4.1: Map of parasitoid collection sites across the Western Cape Province of South Africa using different trapping methods. For the list of the sites, refer to Appendix 1.

In the insectary, all fruit were counted and weighed before being transferred into specially made containers (Copeland et al., 2002). The containers were 2L plastic ice cream containers with cut-out lids replaced with fine mesh (organza material, Fabric Centre, Somerset West, South Africa) which had a gauge small enough to prevent any emerging parasitoids from escaping and allow for air flow and ventilation (Figure 4.2). Two to three centimetres of Malmesbury sand, which was previously

sterilized by being placed in a -20°C freezer overnight, was placed at the bottom of each container to serve as a pupation medium and to absorb juices from the fruit (Mkize et al., 2008). A maximum of five fruits were placed into each container to prevent squashing, but more were added when the fruits were small in size (i.e. grapes). The containers were stored in an insectary room at 25°C ± 2°C on a 16L: 8D light cycle (Mkize et al., 2008).

Fruit containers were monitored for fruit fly or parasitoid emergence every 3-5 days, for up to 5 weeks, by examining each container for a minimum of 10 seconds. To prevent disturbance or damage to the pupae, adult wasps and fruit flies were left to emerge within these containers (Copeland et al., 2002). Adult flies and wasps (dead or alive) were removed, placed in 99% alcohol and stored in a labelled specimen vial and Eppendorf tube, respectively.

Figure 4.2: Modified 2L ice-cream containers with ventilated lids used to rear tephritid fruit flies and their parasitoids from fruits sampled from commercial farms in the Western Cape Province.

Dr Tertia Grové (Agricultural Research Council- ARC-Tropical and Subtropical Crops, Nelspruit, South Africa) collected additional parasitoids in the same manner from multiple host fruit in the Limpopo and Mpumalanga Provinces between December 2012 and December 2016 (Appendix 2).

Sentinel traps

The use of live Medfly hosts to trap parasitoids was investigated by setting out egg- and larva-infested apples as well as exposed Medfly pupae. This method was replicated at three different non-commercial, organic sites (Figure 4.1). The site at Tierhoek farm was an organic apricot (Imperial) orchard, which only sprayed GF-120 for fruit fly control. The Babylonstoren site was a stone fruit

garden where they made use of bucket traps, M3 bait stations and GF-120 for control. At Spier, the trapping site was an abandoned pear orchard where no form of control for any pest was used. The experiment was carried out over 5 weeks from November-December 2016.

Trapping with eggs and larvae

Store-purchased Golden Delicious apples (Food Lovers Market, Stellenbosch) were placed on raised metal mesh racks in a Perspex cage with 7-14 day old sexually mature *C. capitata* adults. Females were allowed to freely oviposit on the apples and were also supplied with sugar-water. After 24 hours, apples required for egg sentinels were placed in the field, while those required for larval sentinels were removed from the adult cage and kept at 25°C for 2 days to allow eggs to hatch. A few infested apples were kept in the lab as a control to rear out flies, thus ensuring the presence of eggs and larvae in apples placed in the field. Egg- and larva-infested apples were transported to the field in a cooler box. A single infested apple was placed on a circular 2L white plastic lid, which was placed in an orange onion-netting trap, and suspended from a branch using a cable tie (Figure 4.3A). Thirty traps of each sentinel stage were hung at each site. At Spier and Tierhoek, one trap was hung in every second tree - spaced about 1m apart - in three different rows. At Babylonstoren, traps were spread evenly across the stone fruit area. Egg sentinel traps were left in the field for 2 days, while the larvae-infested apples were left in the field for 3 days.

Trapping with pupae

For the pupal sentinel traps, a 125ml transparent tub was glued to the centre of the lid in the onion-net trap and two holes were drilled to allow for drainage of any water (Figure 4.3B). About thirty to fifty 2-day old *C. capitata* pupae, obtained from Entomon Technologies (Pty) Ltd (Stellenbosch), were glued to a square of white cardboard, which was stuck to the middle of the tub using a non-toxic glue (Prestick®). Each tub of pupae was set up in the field in the same arrangement as the other sentinel stages, for 3 days. To ensure pupae were alive and healthy, a few pupae were kept in the laboratory and adults were reared out as a control.

Figure 4.3 A simple and inexpensive sentinel trap design, using a bucket lid and onion-netting, holding a *Ceratitis capitata* egg- or larvae- infested apple (A) and pupae glued onto white cardboard (B).

All collected traps (infested apples and pupae) were kept in the insectary at Stellenbosch University at 25 ± 2°C, 16L: 8D. To prevent microbial growth, infested apples were dipped in Sporekill™ (Hygrotech, South Africa) for 1 min, dried with a clean paper towel and then placed individually on a layer of sterile vermiculite in a transparent, medium-sized fast-food container (Figure 4.4). The cardboard containing the pupae was simply removed and placed separately into smaller containers. These containers were pre-prepared by cutting out a large section of the lid and replacing it with organza material to allow for ventilation. Small 2x2cm squares of yellow sticky traps (Chempac, South Africa) were added to the side of each container with cellotape to attract emerging insects and facilitate collecting. Traps were checked at least 2-3 times a week. Sticky traps, which had caught emerged Medflies were removed and replaced with new sticky traps.

Figure 4.4 Transparent and ventilated fast food containers, holding infested apples placed on vermiculite, were used to rear out *Ceratitis capitata* and trapped wasps. A small block of yellow sticky pad was added to each container to facilitate capturing of emerged insects.

The trapping protocol was modified in the second year of the study to improve on the design. Five non-commercial fruit growing sites were selected and only exposed pupae were utilised as sentinel traps (Figure 4.1). The experiment was carried out over five weeks during March-April 2017. A Multilure bucket trap with Biolure and DDVP insecticide was simultaneously set up at each site to monitor fruit fly abundance during the surveying period (Ero, 2009). The non-commercial, organic sites are presented on the map (Figure 4.1). Using the same trap design described above, 1-3 day old Medfly pupae were glued to a piece of white cardboard and centred in the trap. Ten traps were spread out at each site: near fruit trees, close to the ground and in the shade, where possible. The top and bottom of the trap were tied to the tree to prevent the wind from vigorously blowing around the light-weight trap. This experiment was repeated over five weeks, resulting in a total of 50 traps set at each site. Each trap was left out for 3 days before being collected and transported to the Insectary at Stellenbosch University. Cardboard squares holding the pupae were individually transferred to ventilated, clear plastic fast food containers and maintained at a constant temperature of 25 ± 2°C, 16L: 8D. The number of pupae on each trap was recorded to allow for later analysis of non-emergence. The fast food containers were monitored every 2-3 days. Emerging Medflies were removed, counted and discarded, while any emerging wasps were preserved in 99% alcohol.

Preservation of parasitoid wasps

In order to preserve specimens for later identification, all collected specimens were preserved in 99% alcohol and stored in the dark in a 4°C fridge. In collaboration with a taxonomic expert, Dr Simon van Noort (Iziko South African Museum), each specimen was dried using the critical point drying method in the Leica EM CPD300 machine whereby CO_2 and 99% alcohol were exchanged twenty times. Each individual parasitoid wasp was subsequently mounted on the tip of a black triangular acid-free card (Peters Papers, Cape Town) using water-soluble glue. An information label was prepared and a museum catalogue number provided for each pinned specimen (Figure 4.5). All specimens have been deposited at the Iziko museum. For expert identification, various angles of some of the specimens were imaged and measurements were included on each image using Leica Application Suite v4.4 with an 18% grey card as a standard background. Completed images were sent for further expert identification and images and information were uploaded to Waspweb (www.waspweb.org).

Figure 4.5 Pinned wasp specimens labelled with collection details and a museum catalogue number.

Identification of parasitoid wasps

All collected flies and wasps were identified using keys (Goulet and Huber, 1993; Prinsloo and Eardley, 1985a; Virgilio et al., 2014; www.paroffit.org). For molecular identification, 33 wasp specimens were selected, based on morphospecies sorting. Non-destructive DNA extraction was performed using a DNeasy blood and tissue extraction kit (Qiagen©) following the manufacturer's instructions and stored at -20°C. The amount of DNA (ng/µl) in the final product was measured for each specimen using a Spectrophotometer ND-1000 (NanoDrop Technologies).

Amplification of a portion of the COI gene was attempted using five different primer pairs for the unknown wasp specimens (Table 4.1). PCR conditions for primer pair 1 were an initial denaturation of 94°C for 5min; then 35 or 40 cycles of 94°C for 30s, annealing at 46,52,55,56 or 58°C for 30s, extension at 72°C for 1min; and a final elongation of 72°C for 10min. Primer pairs 2-5 were run at an initial denaturation of 96°C for 5min; then 30,35 or 40 cycles of 96°C for 30s, annealing at 51°C for 30s, extension at 72°C for 50s; and a final elongation of 72°C for 5min. For each PCR run, a positive (known specimen) and negative (deionized water) control was included to ensure that there was no contamination or error made during the process.

PCR amplification products were run on a 1% agarose gel to confirm successful amplification (Ashfaq et al., 2010). Post-PCR purification was undertaken using the NucleoFast® Purification System (Macherey-Nagel). Sequencing was performed with the BigDye Terminator V1.3 sequencing kit (Applied Biosystems), followed by the use of electrophoresis on the 3730 × l DNA Analyser (Applied Biosystems) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University).

Table 4.1 Primer pairs used to amplify the COI gene region of the parasitoid specimens collected in the present study.

Pair	Primer	Sequence (5'-3')	Reference / Source
1	LCO-1490 HCO-2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994
2	C1-J-2183 TL2- N-3014	CAACATTTATTTTGATTTTTTGG TCCAATGCACTAATCTGCCATATTA	Simon et al., 1994
3	C1-J-2183 TL2- N-3014Bee	CAACATTTATTTTGATTTTTTGG TTCAATGCACTTATTCTGCCATATTA	Simon et al., 1994
4	C1-J-2195 TL2- N-3014	TTGATTTTTTGGTCATCCAGAAGT TCCAATGCACTAATCTGCCATATTA	Simon et al., 1994
5	TL2- N-1718 C1-J-2329Bee	GGAGGATTTGGAAATTGATTAGTTCC ACTGTAAATATGTGATGTGCTCA	Simon et al., 1994

Results and Discussion

Collection of fruit fly infested fruit

In total, fruits weighing 125.78kg were collected during this study. They comprised 20 different fruit types, and 568 emerged wasps grouped into 33 different morphospecies (Table 4.2). The largest proportion of the wasps was reared from the various host fruits whilst others were incidentally collected from a soil sample and a fruit fly bucket trap using a Biolure attractant (Table 4.3). The total number of fruit collected varied across the Provinces. In the Western Cape, fruits were collected from commercial orchards and included larger fruits such as apples, pears, plums and peaches. In the other two Provinces, wild fruits were collected from alternative fruit fly host plants and were smaller sized fruit such as marula, rose apples and water berries (Appendix 2). Very few parasitoids and fruit flies emerged from the Western Cape fruit, relative to the other Provinces, and this may indicate the negative effect of chemical controls on parasitoids despite their successful control of fruit flies (Adan et al., 2011). The high abundance of parasitoids reared from Mpumalanga and Limpopo relate to the high abundance of fruit flies, which make use of indigenous fruits as alternate hosts (Grové et al., 2017). The smaller size of these fruits may have also made eggs and burrowing larvae more accessible to the short ovipositor of the female parasitoids (Feder, 1995).

Table 4.2 Number of fruit flies and parasitoids obtained from the fruit sampling carried out in the three South African Provinces.

Province	No. of fruit	Mass (kg)	No. of fruit flies	No. of parasitoids	No. of morphospecies
Western Cape	1400	103.58	216	17	11
Mpumalanga	1086	12.28	986	319	8
Limpopo	2048	9.92	1496	232	14
TOTAL	4534	125.78	2698	568	33

Table 4.3 Taxonomic identification for each of the 33 wasp morphospecies. Their catalogue number, common name of host fruit from which they were reared, and species of fruit fly (*Ceratitis* and *Bactrocera*) that emerged from the same fruit is also presented. Primer pairs tested are indicated for each specimen.

WESTERN CAPE							
Superfamily	**Family**	**Subfamily**	**Species**	**Catalogue number**	**Host**	**Fruit fly species**	**Primer pair tested**
Chalcidoidea	Encrytidae		1	P065283	Pear	*C. capitata*	1; 2; 4
Chalcidoidea	Encrytidae		2	P065284	Pear	*C. capitata*	2; 4
Cynipoidea	Figitidae	Eucoilinae	*Leptopilina spp.*	P065278	Apple	*C. capitata*	1; 2; 4
Cynipoidea	Figitidae		1	P065277	Plum	*C. capitata*	1; 2; 4
Cynipoidea	Figitidae		2	P065281	Plum	*C. capitata*	1; 2; 4
Ichneumonoidea	Braconidae	Alysiinae	*Alysia manducator*	P065460	Bucket trap	*C. capitata*	1; 2; 3; 4; 5
Protrupoidea				P065280	Soil	-	1; 2; 4
Unknown				P065287	Apple	*C. capitata*	1; 2; 4
Unknown				P065290	Apple	*C. capitata*	1; 2; 4
Unknown				P065291	Apple	*C. capitata*	1; 2; 4
Unknown				P065293	Vine	*C. capitata*	2; 4
LIMPOPO							
Superfamily	**Family**	**Subfamily**	**Species**	**Catalogue number**	**Host**	**Fruit fly species**	**Primer pair tested**
Chalcidoidea	Eulophidae			P065513	Guava	*C. capitata* *C. rosa* *B. dorsalis*	1; 2; 3; 4; 5
Cynipoidea	Figitidae	Eucoilinae	1	P065478	Enkeldoring noem-noem	*C. capitata*	1; 2; 4
Cynipoidea	Figitidae	Eucoilinae	2	P065497	Guava	*C. capitata* *C. rosa*	1; 2; 4
Evanioidea	Evaniidae			P065484	Enkeldoring noem-noem	*C. capitata*	1; 2; 4
Ichneumonoidea	Braconidae	Euphorinae	1	P065486	Cape ash	*C. capitata*	1; 2; 4
Ichneumonoidea	Braconidae		3	P065500	Brown ivory	*C. capitata*	1; 2; 4

Ichneumonoidea			3	P065517	Marula	*C. cosyra*	2; 4
Unknown				P065506	Enkeldoring noem-noem	*C. capitata*	1; 2; 4

MPUMALANGA

Superfamily	Family	Subfamily	Species	Catalogue number	Host	Fruit fly species	Primer pair tested
Chalcidoidea	Eulophidae			P065473	Strawberry guava	*C. rosa*	1; 2; 4
Chalcidoidea				P065475	Feijoa	*C. rosa*	1; 2; 4
Chalcidoidea				P065488	Waterberry	*C. capitata* *C. rosa*	1; 2; 4
Ichneumonoidea			1	P065468	Strawberry guava	*C. capitata* *C. rosa*	1; 2; 3; 4; 5
Ichneumonoidea			2	P065509	Rose apple	*C. rosa*	1; 2; 4
Ichneumonoidea	Braconidae		1	P065469	Marula	*C. cosyra*	1; 2; 4
Ichneumonoidea	Braconidae		2	P065495	Waterberry	*C. capitata* *C. rosa*	1; 2; 4
Ichneumonoidea	Braconidae		1 (repeat)	P065502	Rose apple	*C. rosa*	1; 2; 4
Ichneumonoidea	Braconidae		2 (repeat)	P065503	African mangosteen	*C. rosa*	1; 2; 4
Ichneumonoidea	Braconidae		3 (repeat)	P065510	Rose apple	-	2; 4
Ichneumonoidea	Braconidae	Alysiinae	1	P065466	Rose apple	*C. rosa*	1; 2; 3; 4; 5
Ichneumonoidea	Braconidae	Alysiinae	2	P065463	Strawberry guava	*C. rosa* *C. cosyra*	1; 2; 4
Ichneumonoidea	Braconidae	Alysiinae	3	P065490	Rose apple	*C. rosa*	1; 2; 4
Proctotrupoidea	Diapriidae			P065482	Feijoa	*C. rosa*	1; 2; 4

Fruit sampling was the most appropriate method for recovering parasitoid wasps and fruit flies, but required sampling large amounts of fruit to obtain significant results. In North-western Argentina, fruit collection was conducted over a 5-year period, yielding five species of parasitoid wasps (Ovruski et al., 2004) and a shorter study in Australia found parasitoids in only 9% of the collected fruit (Spinner et al., 2011). Several studies rearing wasps from collected fruit have also been conducted in various African countries (Copeland et al., 2004; Vayssières et al., 2011; Wharton et al., 2000) and in South Africa (Manrakhan et al., 2010; Mkize et al., 2008; Neuenschwander, 1982). Although fruit sampling is the most effective means of recovering parasitoids, it requires a large amount of space for rearing and is labour and time intensive. Certain host fruits are also not available throughout the year or vary in availability which may also require multiple site visits (Copeland et al., 2009). Furthermore, the simultaneous emergence of fruit flies and other insects (beetles, drosophilid flies, moths) which could also be the wasps' potential hosts, may limit the ability to confirm from which insect the parasitoid wasps have emerged. This however does not apply for wasps emerging from pupae, as pupae could be sifted from the vermiculite and held in a separate container fitted with glass tubes to which the wasps and flies would fly due to attraction by light (Ovruski et al., 2004).

It is likely that separation of fruit fly pupae from the collected fruit, could provide useful data in confirming fruit fly specific parasitoids (Ali et al., 2016; Badii et al., 2016). However, this was not carried out in the present study, nor in other South African studies (Manrakhan et al., 2010; Neuenschwander, 1982). In a study by Wharton et al. (2000) where pupae were isolated and held individually, additional handling resulted in reduced emergence of flies and parasitoids. It should be noted that the pupa from which the parasitoid emerges would still require identification in order to link the wasp to a host, and this may be challenging for non-experts (Garcia and Corseuil, 2004). Dissection of the fruit to separate larvae would also facilitate specification of wasp-host relationships, but is labour intensive and requires optimized larval-rearing conditions (Montoya et al., 2016; Spinner et al., 2011).

Fruit sampling also has the disadvantage of excluding sampling for pupal parasitoids (see also Manrakhan et al., 2010). Along with the sampling of fruit, Wharton et al. (2000) released third instar larvae as sentinels directly into the soil in an experimental field and after 3-7 days recovered 55% of the pupae (Wharton et al., 2000). Although they were able to obtain pupal parasitoids in this way, the difficulties in recovering pupae (and potential escape of this pest into the wild) is a drawback for such a method. Therefore, surveying for target-specific parasitoids should be more easily achieved by trapping them with sentinel eggs, larvae and pupae, using the methods suggested in the current study.

Sentinel traps

The use of sentinel trapping is a useful way of collecting parasitoid wasps parasitizing the target host (Harbi et al., 2015). No wasps were obtained using this method in the present study, but the methodology provides a promising technique which, with more extensive trapping, could result in Medfly life stage-specific parasitoids (Ero, 2009).

The use of apples for the sentinel trapping served as a robust fruit, withstanding environmental factors and not rotting or disintegrating in the laboratory during the rearing period. Other studies have also made use of apples for sentinel trapping (Ero, 2009; Harbi et al., 2015; Santiago et al., 2006). However, Feder (1995) found that larger host fruit, such as apples, might provide better physical protection for developing tephritid larvae from ovipositing female parasitoids. Therefore, using smaller sentinel fruit may potentially increase collection of parasitoids. A similar methodology to our study was used for trapping of *B. dorsalis* egg parasitoids, by allowing 20 *B. dorsalis* females to infest a papaya over 24 hours (Eitam and Vargas, 2007). Natural infestation was also used in a study surveying for *D. suzukii* parasitoids, whereby strawberries were placed in a cage with two female *D. suzukii* flies for 24 hours, before maintaining the fruit at 25°C to allow larval development (Gabarra et al., 2015). In the study by Harbi et al. (2015), apples were artificially infested by placing late second instar larvae into pre-bored holes. This allowed them to know the number of sentinel insects set out in the field and allowed them to determine percentage parasitism.

Sentinel traps were set out in non-commercial, organic fruit-growing areas with the expectation that natural enemies might be in greater abundance where no insecticides were used (Harbi et al., 2015). High fruit fly numbers were also expected in these orchards, but trapping data revealed only 22 fruit flies during the 5-week experiment. A study in Italy using *D. suzukii* to trap parasitoid wasps had to run their experiment for over two months to record any success, but eventually obtained high parasitism (Rossi Stacconi et al., 2013). Therefore, extending the trapping over several months will provide more comprehensive data, but limited available time prevented extensive trapping within the current study. However, the trap design used provides a simple and inexpensive trap for surveying fruit fly parasitoids and this is important as cost and assemblage remains an important consideration in any experiment (Verghese et al., 2002).

Investigation of other possible means of trapping parasitoids of fruit-infesting Tephritidae has been studied elsewhere, for example, by using visual and olfactory stimuli. For instance, fruit odour, simulating host fruits, attracted females of a larval parasitoid *Diachasmimorpha longicaudata* (Ashmead) to sticky traps (Messing and Jang 1992). Vargas et al. (1991) found that the colours yellow and white were attractive to *B. dorsalis* and its' parasitoids, and could be incorporated into a

monitoring method for both. However, these still do not provide direct host-parasitoid links, but only associations. Therefore, the use of sentinel traps may provide the most effective method.

Identification of parasitoid wasps

Identification of each morphospecies to the lowest level possible is presented in Table 4.4, but requires expert verification. Only one specimen (P065460) was morphologically identified by an expert, Dr Francisco Javier Peris Felipo (Syngenta), as *Alysia manducator* (Panzer) (Braconidae: Alysiinae) and specimen images have been uploaded to Waspweb (Figure 4.6). This species is attracted to the smell of meat and is a known parasitoid of blowflies (Reznik et al., 1992). Its presence in the fruit fly bucket trap may thus have been due to the strong smell of the Biolure attractant, rather than an association with the fruit flies. Accurate identification is the basis of a successful biological control programme and will ensure that the time and money invested into further research is validated (Gariepy et al., 2007; Hebert et al., 2003). However, it appears that the relevant expertise on this group of parasitoids is lacking for the South African fauna and many species still need to be described. Families most often attacking fruit-infesting tephritid fruit flies include Braconidae, Chalcididae, Diapriidae, Eulophidae, Eupelmidae, Eurytomidae, Figitidae (Eucoilinae), Ichneumonidae, and Pteromalidae, with four of these families having been documented in the present study.

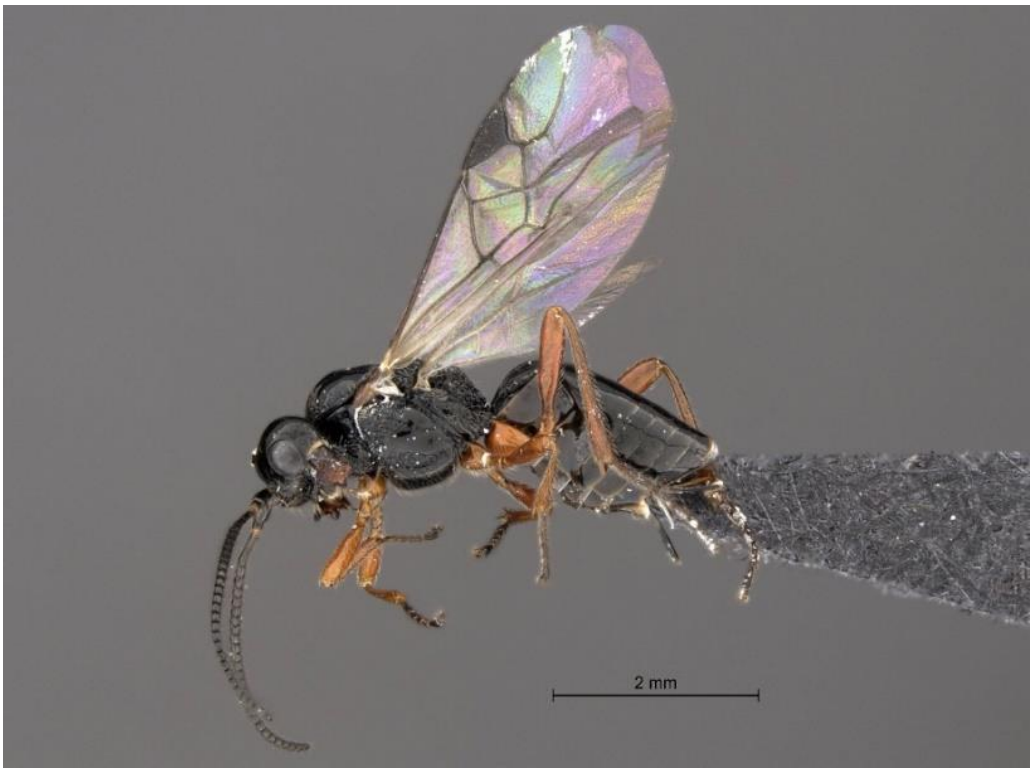

Figure 4.6 Lateral view of *Alysia manducator* (Panzer) (Braconidae: Alysiinae) which was found in a yellow Multilure bucket trap together with *Ceratitis capitata*. More images of this specimen are available on www.waspweb.org.

DNA barcoding is a simple and powerful tool which can be used to accurately identify species (Hebert et al., 2003; Jenkins et al., 2012; Jinbo et al., 2011). DNA was successfully extracted from the 33 wasp specimens, although extracted DNA was low, ranging from 1.4-9.8 ng/µl. Despite various attempts at optimising different primer combinations (Table 4.1) for the different wasp specimens, limited success was only achieved using the primers C1-J-2195 and TL2-N-3014 on samples P065460, P065466, P065468 and P065513 (Figure 4.7). This primer pair was successfully used to amplify a fragment of the COI gene of a mealybug parasitoid (Ashfaq et al., 2010). Blasting the sequenced PCR products on Genbank and BOLD did not result in a 100% identification match to any sequences in the databases. Top results, with 80-95% matches, were all species of Hymenoptera, but it was not possible to make identifications further than family level (Table 4.4). The other specimens remained unamplified and possible reasons for this could be attributed to low DNA quality, incorrect concentrations of buffer components and/or cycling conditions (Roux, 2009). Quantity of extracted DNA has been found to significantly influence the likelihood of sequencing the COI fragments from braconid specimens and may have been an issue in our study (Andersen and Mills, 2012). Increasing the number of cycles, DNA template or initial denaturing temperature in the PCR may provide better amplification of the other specimen DNA (Roux, 2009). Variations of the buffer components and primers may provide optimal amplification conditions and use of enhancing agents, such as Bovine serum albumin, may increase specificity and yield of the PCR product (Hajibabaei et al., 2005; Roux, 2009; Wagener et al., 2006). Furthermore, testing other primer combinations and possibly designing new primers should be considered for future studies, as identification of the collected wasps could provide valuable insight on the current parasitoid complex in South Africa.

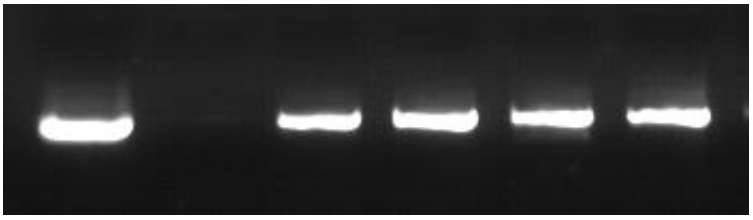

Figure 4.7 DNA bands of the positive control and four wasp specimens viewed under UV light.

Among the specimens likely to be braconids, some were morphologically identified as part of the subfamily Alysiinae due to their mandibles not touching when closed (Wahl and Sharkey, 1993; Figure 4.7). This is one of three braconid subfamilies containing species that attack tephritid fruit flies, with the other two being Opiinae and Braconinae (Wharton and Yoder, n.d.). Parasitoids of the Alysiinae are classified as koinobiont endoparasitoids because their offspring live within the egg or larval host and allow the host to continue development, while feeding and growing within, eventually emerging from the pupa (Bonet, 2008). Other specimens sampled belong to the Eucoilinae, based on the distinct raised plate on their scutellum (Figure 4.8A). This is a subfamily of Figitidae based on the classification by Ronquist (1995). Known tephritid-attacking eucoilines belong to the genera

Aganaspis and *Odontosema*, which have been reared from isolated puparia (Wharton and Yoder, n.d.). Two species of *Aganaspis* are utilized in tephritid biological control efforts. *Aganaspis pelleranoi* Brèthes is a parasitoid of *C. capitata*, but achieves better control of *Anastrepha fraterculus* (Wiedemann), for which research on mass-rearing is on-going in South America (Gonçalves et al., 2016). *Aganaspis daci* Weld is being investigated as a control for *C. capitata*, especially under Mediterranean climatic conditions (de Pedro et al., 2017). To date, no species of this genus have been recorded in South Africa (Appendix 2), but one specimen has been recorded from the Central African Republic (van Noort et al., 2015). Species of the genus *Leptopilina* are well-known parasitoids of Drosophilidae (Nordlander, 1982) and the wasp reared from apples in this study is thus more likely to have emerged from drosophila flies that were also present in the fruit (Figure 4.7A). Eulophidae were also sampled in this study, and there are species of this family that are specific parasitoids of tephritid fruit flies. The eulophid *Tetrastichus giffardianus* was reared from fruit flies infesting coffee berries during a recent study in South Africa (Manrakhan et al., 2010).

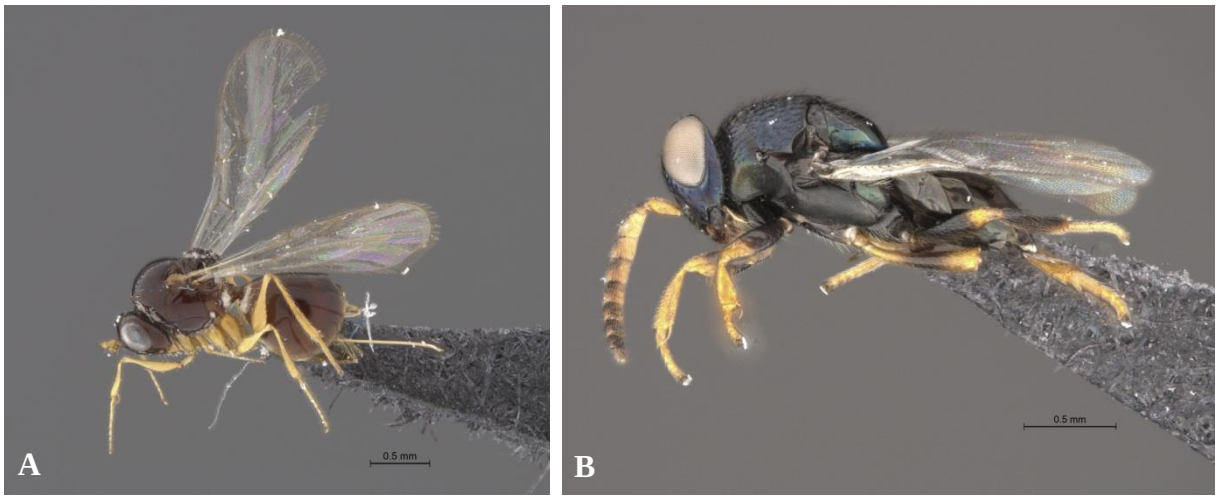

Figure 4.8 Imaged specimens belonging to the families Figitidae (A) and Encrytidae (B).

Conclusion

This study investigated the parasitoid wasps associated with fruit fly pests in South Africa. Further work is required not only in the form of fruit collections, but specifically surveying for host- and life stage-specific parasitoids. This is important, as it is crucial in knowing whether they are effective biological control agents against target pests, such as the Medfly. Moreover, many of the wasps found in this study remain unidentified, and further molecular work, such as designing primers and better optimizing protocols, should be considered as identifying these wasps would provide valuable information for South Africa and may feed into future biological control programmes (Jenkins et al., 2012). The use of sentinel trapping could be useful for identifying Medfly-specific parasitoids and future studies should consider areas of known high fruit fly abundance and infestation for these traps,

including areas where small indigenous fruits serve as alternative hosts for fruit flies (Grové et al., 2017). To better optimize this technique, traps should be set out over more seasons to include the influence of climatic variability on parasitoid abundance (Sivinski et al., 1998), be repeated over a longer period of time, and should consider the use of smaller fruit for egg/larval parasitoid retrieval (Feder, 1995). Further efforts are important, as a new agent would greatly enhance the control strategies available for fruit fly management, as part of an IPM programme, in South Africa.

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CHAPTER 5

General discussion

The continuous demands from export markets and awareness of risks associated with chemical pesticide use has increased the urgency of finding alternative methods for insect pest control (Dolinski and Lacey, 2007; Lacey and Shapiro-Ilan, 2008). Low yield, poor crop quality, and the inability to meet phytosanitary standards can lead to market exclusion and thus socio-economic problems for African producers (Ekesi et al., 2016). These problems are a reality due to the presence of fruit flies, specifically those that are polyphagous, having a broader host range, thus threatening access to multiple markets and affecting global fruit trade (White and Elson-Harris, 1992). The Mediterranean fruit fly (Medfly), *Ceratitis capitata*, is a notorious pest across the world and is dominant in many areas of South Africa (Du Toit, 1998). Its ability to use a multitude of fruits and vegetables as host allows it to persist and remain problematic, causing extensive damage and requiring expensive controls. This study aimed to investigate biological control options in the form of entomopathogenic nematodes (EPNs), entomopathogenic fungi (EPF) and parasitoid wasps, which could serve as environmentally friendly and sustainable alternative controls. This included surveying for local EPN and EPF isolates, screening pathogenicity of entomopathogens against Medflies under optimal and soil conditions, as well as surveying for parasitoids attacking fruit-infesting fruit flies.

Entomopathogenic nematodes, which are natural inhabitants of soil, contain symbiotic bacteria in their gut, allowing them to utilize insects as hosts (Griffin et al., 2005; Stock, 2015). However, in South Africa, there has only been one study looking at the potential of EPNs as a Medfly control (Malan and Manrakhan, 2009). All local EPNs screened were pathogenic against the third instar Medfly larvae, which is the life stage that enters the soil to pupate (Thomas et al., 2010). More specifically, this study highlighted the potential of the local species *Heterorhabditis noenieputensis* as a candidate biological control agent. When third instar larvae were directly inoculated with EPNs, this species caused a mortality of over 80% at a concentration of 100IJs / 50µl, and maintained significantly higher control compared to the other EPNs, at the lower concentration of 50IJs / 50µl. Exposure of third instar larvae to a container of moist sand, which had been inoculated with *H. noenieputensis* 24 hours earlier, resulted in 94-100% being infected and killed. Importantly, many died as pupae, which had been infected as larvae, highlighting the potential of such a virulent species to control the soil life stages of this pest and prevent emergence of adult flies. If successful, controlling the soil life stages has several advantages: fewer chemical sprays would be required, thus reducing chemical reliance and residue on fruit; fewer adults would emerge and there would be less oviposition and damage to host fruit; and control of adults would also be easier (and cheaper), because population numbers would have been suppressed. However, a drawback of a larval-attacking species, such as *H. noenieputensis*, is that fully developed infective juveniles may not be able to exit the

impenetrable puparium (Malan and Manrakhan, 2009). Shorter application intervals of such an EPN may thus be required to sustain its' population. This problem may be overcome by a species such as *S. yirgalemense*, which, in this study, infected Medflies emerging from the puparium, which then died as adults. Infective juveniles would be able to exit the softer exoskeleton of the adult fly, and the distance the adult is able to fly before dying may further spread the EPN, increasing its persistence in the field and potential control. Commercialization of *S. yirgalemense* requires extensive research, time and money (Ferreira et al., 2016), but its ability to control multiple pests, including the Medfly, should enhance interest and use by growers, therefore paying off the initial investment. Overall, there is potential for the use of effective EPNs in an integrated pest management system for fruit fly (Malan and Manrakhan, 2009).

Similar to EPNs, only one previous study has been conducted, testing EPF against the Medfly in South Africa (Goble et al., 2011). Entomopathogenic fungi also reside in soils and when they encounter a susceptible insect, they penetrate and kill it, using it to propagate new conidia. When conditions are less favourable (e.g. low moisture, no hosts), they form resting spores and are able to persist in the soil for long periods of time (Inglis et al., 2001; Wraight et al., 2007). This study used the dipping method to directly inoculate third instar larvae either with local isolates or commercial EPF products to investigate pathogenicity (Inglis et al., 2012). The ability of the three commercial products, Broadband®, EcoBb® and Meta69, to cause at least 50% mycosis suggests that Medfly contact with these products in the field could cause death, and thus be a form of control. Such a finding is relevant as these products, which are readily available, could assist in regulating pest populations other than what they are registered for. However, their use is limited to existing formulations which may not be practical to use in orchards for fruit fly control (Goble, 2009).

A local EPF isolate belonging to the *Metarhizium anisopliae* complex, MJ06, trapped in a soil sample by using a Medfly larva as bait, was highly pathogenic when tested against the Medfly in screenings. MJ06 showed promising results in varying degrees of moisture, and offered good control in soil bioassays (>60%). Another local isolate, which originated from a woolly apple aphid (*Eriosoma lanigerum*), *Beauveria bassiana* (6756), also caused high percentage mortality and mycosis of Medfly (70%), but percentage mycosis was significantly lower in the experiment with low moisture (10%). Establishing optimal abiotic requirements of EPF in the field is necessary, and will assist in ensuring efficiency as well as correct daily and seasonal timing of applications (Inglis et al., 2001; Maniania and Ekesi, 2016). This study demonstrates that application of EPF to soil in orchards could offer control against third instar larvae, pupae and the adults emerging from the soil. The high mycoses caused on adult fruit flies may further favour the recycling and spread of conidia in the environment, thus augmenting the control agent (Ekesi et al., 2007; Goble, 2009). Further investigation of isolate MJ06 may afford a virulent Medfly control to incorporate into an IPM system.

Parasitoids are generally more host-specific, having co-evolved with their host, and actively search for eggs or larvae in fruit or pupae in the soil, depending on the parasitoid species (Mohamed et al., 2016). This study's survey of fruit fly parasitoids produced many wasps from a multitude of fruits, but difficulties in identification has limited the new knowledge about them and also prevented the identification of their insect host. Attempts to obtain Medfly specific parasitoids by baiting with Medfly eggs, larvae and pupae were unsuccessful, although modification and optimization of the methodology could result in Medfly-specific biological control agents (Ero, 2009; Harbi et al., 2015). The inexpensive and simple onion net trap used in this study provides a good starting point for future sentinel trapping.

According to Mohamed et al. (2016), there are plans to introduce two well-known fruit fly parasitoids into South Africa for the control of the recent invader, *Bactrocera dorsalis*. However, the use of introduced natural enemies does not come without risks: unpredictable host shifts to non-target organisms, outcompeting of native agents, and possible attacks on introduced organisms by local predators cannot be excluded. It is important to try to mitigate these potential risks as far as possible and also highlight the importance of an expanded search for local natural enemies, such as the undertakings of this study, for use as effective biological control agents for all Medfly life stages (Ekesi et al., 2016).

The positive attributes of biological control agents or "bio-pesticides", such as the ability to self-sustain thus reducing augmentative applications required, ease of application, no restrictive residues and overall safety, are giving them increased popularity (Olson, 2015). Their use can offer highly favourable returns and optimized application timing can reduce overall reliance on chemical pesticides. Because use of natural enemies involves utilizing a natural process, they would be able to sustain themselves using the pests as hosts for reproduction. This makes them available throughout the year which can also offer sanitation and control during the low periods of fruit production (Goble, 2009).

However, growers can't rely on a single product, and the integration of biological control agents into IPM is necessary to maximize efficiency (Lacey et al., 2015). An IPM approach refers to the selection and use of pest control methods that are economically, environmentally and socially favourable (Blake et al., 2007). Assessing the combined effects of biological control with chemical controls is also necessary as it could reduce the dependency and amount of chemicals that are used (Muriithi et al., 2016). Moreover, ensuring synergistic effects between different methods, including biological control agents, is important for efficient control as incompatibility will deem the use of such natural agents pointless (Stokwe, 2016). Ekesi et al. (2011) found that the use of GF-120 (protein bait with spinosad) with *Metarhizium anisopliae* provided greater control of the fruit fly *B. dorsalis* than when either was used in isolation. A study investigating the effect of an EPF, *Metarhizium anisopliae,* on

the fruit fly parasitoids *Psyttalia concolor* and *P. cosyrae*, found no harmful effects caused to the parasitoids, highlighting that the EPF could offer fruit fly control without negatively affecting the natural enemies (Ekesi et al., 2005). Furthemore, parasitoids are a well-suited control which could be used in conjunction with a sterile insect technique (SIT) programme (Wong et al., 1992). In South Africa, use of SIT against Medfly is showing success and could be enhanced by the simultaneous release of parasitoid wasps attacking the immature life stages of the pest. Further research in this regard, using the candidates highlighted in this study, are required.

Lastly, novel methods used in this study should be considered in future biological control studies. The first was the baiting of soil samples with larvae of the target host, by placing diet on a plate on the soil surface. This allowed larvae to naturally enter into the soil and also provided a means of isolating Medfly-specific entomopathogens. The second was the use of bioassay plates to screen EPF pathogenicity. This technique prevented horizontal transmission of conidia between insects, thus providing a stringent method for assessing true pathogenicity. The third was the simple and inexpensive design of the onion-netting trap for sentinel trapping of parasitoids. Use of this design would be valuable in identifying parasitoids that are specific to different life stages of the targeted host.

In conclusion, very little research has been conducted in South Africa on the use of local entomopathogens and parasitoids for control of the Medfly and this study provides baseline results giving guidance to future studies. Field trials against the Medfly are required for *H. noenieputensis* and isolate MJ06, and a combination of the two should also be considered for investigation. Extensive soil sampling, especially using the target host as bait, should be considered in all future studies. Furthermore, a large-scale study using sentinel traps is suggested to locate and identify Medfly-specific parasitoids. The integration of EPNs, EPF and parasitoids into an IPM system would ultimately provide sustainable and effective control of this target pest. Thus, this study has provided necessary groundwork for future biological control efforts against the Mediterranean fruit fly in South Africa.

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Appendix 1

Site information of the farms and home gardens where fruit sampling and sentinel trapping was conducted in the Western Cape Province between January 2016 and April 2017. The relevant code used in Figure 4.1 is also provided.

FRUIT SAMPLING				
Farm name	Area	Fruit orchards sampled	Map code	GPS co-ordinates
Welgevallen	Stellenbosch	Pears, Citrus, Apples, Plums	7	S33° 56' 54.78" E18° 52' 18.89"
Fransmanskraal	Stellenbosch	Fortune Plums, African Delight Plums	6	S33° 54' 38.35" E18° 47' 56.21"
Baldric Farm	Stellenbosch	Panama Apples, Forelle Pears	5	S33° 55' 59.33" E18° 57' 31.21"
Jagerskraal	Prince Alfred Hamlet	September Yummy Plums, Kakamas Peaches	2	S33° 17' 15.33" E19° 20' 13.87"
Vrolikheid	Tulbagh	Forelle Pears	1	S33° 13' 31.18" E19° 08' 29.04"
SENTINEL TRAPPING – all life stages				
Site name	Area	Site	Map code	GPS co-ordinates
Tierhoek	Robertson	Organic farm	3	S33° 42' 43.64" E19° 47' 21.27"
Babylonstoren	Simondium	Organic farm	4	S33° 49' 21.37" E18° 55' 47.99"
Spier	Stellenbosch	Abandoned pear orchard	9	S33° 58' 46.00" E18° 47' 22.90"
SENTINEL TRAPPING – pupae only				
Site name	Area	Site	Map code	GPS co-ordinates
Welgevallen	Stellenbosch	Home garden	7	S33° 56' 54.78" E18° 52' 18.89"
Private residence	Stellenbosch	Home garden	8	S33° 58' 26.01" E18° 46' 37.37"
Spier	Stellenbosch	Abandoned pear orchard	9	S33° 58' 46.00" E18° 47' 22.90"
Private residence	Somerset West	Home garden	10	S34° 03' 14.90" E18° 49' 37.69"
Hathersage	Strand	Abandoned plum orchard	11	S34° 04' 56.18" E18° 51' 54.63"

Appendix 2

Detailed information of host fruit, fruit flies and parasitoid wasps collected from various areas across three South African Provinces.

Limpopo						
Area	**GPS**	**Host fruit**	**No. of fruit**	**Mass (kg)**	**Number of fruit flies**	**Number of wasps**
Letsitele	23° 39.402'S 30° 40.258'E	Simple spine num-num	100	0,2397	188	5
Levubu	23° 5.126'S 30° 16.985'E	Cape-ash	328	0,421	125	8
Thohoyandou	22° 52.712'S 30° 28.911'E	Marula	57	1,2597	16	7
Levubu	23° 5.126'S 30° 16.985'E	Guava	40	3,428	111	40
Letsitele	23° 39.402'S 30° 40.258'E	Brown ivory	355	0,2843	201	109
Letsitele	23° 39.402'S 30° 40.258'E	Brown ivory	218	0,1744	37	25
Letsitele	23° 39.402'S 30° 40.258'E	Brown ivory	752	0,6107	613	2
Letsitele	23° 39.402'S 30° 40.258'E	Simple spine num-num	63	0,1383	56	14
Marobeng	23° 52.775'S 29° 24.661'E	Guava	30	1,2218	121	7
Thohoyandou	22° 52.712'S 30° 28.911'E	Marula	48	0,8773	12	7
Thohoyandou	22° 52.712'S 30° 28.911'E	Marula	57	1,2597	16	8
Mpumalanga						
Area	**GPS**	**Host fruit**	**No. of fruit**	**Mass (kg)**	**Number of fruit flies**	**Number of wasps**
Nelspruit	25° 27.104'S 30° 58.161'E	Strawberry Guava	19	0,141	24	2
Nelspruit	25° 27.104'S 30° 58.161'E	Rose apple	63	2,0615	65	22
Nelspruit	25° 27.104'S 30° 58.161'E	Strawberry Guava	85	0,2073	90	6
Nelspruit	25° 27.104'S	Strawberry	149	0,4105	13	2

	30° 58.161'E	Guava				
Kingstonvale	25° 27.104'S 30° 58.161'E	Water berry	150	0,22	10	5
Nelspruit	25° 27.104'S 30° 58.161'E	Marula	50	1,1266	129	3
Nelspruit	25° 27.104'S 30° 58.161'E	African mangosteen	73	0,5165	69	16
Nelspruit	25° 27.104'S 30° 58.161'E	Feijoa	26	0,5665	59	9
Nelspruit	25° 27.104'S 30° 58.161'E	Strawberry guava	15	0,1077	34	4
Nelspruit	25° 27.104'S 30° 58.161'E	Water berry	100	0,2438	18	26
Nelspruit	25° 27.104'S 30° 58.161'E	Rose apple	63	2,0615	65	32
Nelspruit	25° 27.104'S 30° 58.161'E	Wild mango	20	1,0791	110	3
Nelspruit	25° 27.104'S 30° 58.161'E	African mangosteen	38	0,357	20	20
Nelspruit	25° 27.104'S 30° 58.161'E	African mangosteen	38	0,357	20	6
Burgershall	25° 27.104'S 30° 58.161'E	Stemfruit	55	0,4614	20	11
Nelspruit	25° 27.104'S 30° 58.161'E	Strawberry guava	50	0,2798	146	5
Nelspruit	25° 27.104'S 30° 58.161'E	Rose apple	10	0,288	6	20
Nelspruit	25° 27.104'S 30° 58.161'E	Rose apple	48	1,5912	88	103
Nelspruit	25° 27.104'S 30° 58.161'E	Rose apple	34	0,2	0	24

Western Cape

Area	GPS	Host fruit	No. of fruit	Mass (kg)	Number of fruit flies	Number of wasps
Tulbagh	33° 16.35'S 19° 10.14'E	Plums	10	0,643	8	0
Tulbagh	33° 16.35'S 19° 10.14'E	Plums	12	0,506	1	0
Tulbagh	33° 16.35'S	Peaches	7	0,455	0	0

	19° 10.14'E					
Tulbagh	33° 16.35'S 19° 10.14'E	Peaches	10	0,208	1	0
Stellenbosch	33° 55.328'S 18° 48.729'E	Plums	50	1.7	54	0
Stellenbosch	33° 55.328'S 18° 48.729'E	Plums	50	1,602	4	1
Stellenbosch	33° 55.328'S 18° 48.729'E	Plums	50	3,695	0	0
Stellenbosch	33° 55.328'S 18° 48.729'E	Plums	50	4,459	0	0
Stellenbosch	33° 55.252'S 18° 55.802'E	Apples	55	6,105	3	0
Stellenbosch	33° 55.252'S 18° 55.802'E	Apples	50	5,172	0	2
Stellenbosch	33° 55.252'S 18° 55.802'E	Pears	50	5,042	28	0
Stellenbosch	33° 55.252'S 18° 55.802'E	Pears	50	4,300	60	8
Stellenbosch University	33° 56.864'S 18° 51.705'E	Kei Apples	40	0,433	0	0
Stellenbosch	33° 56.864'S 18° 51.705'E	Pears	100	5,919	28	0
Stellenbosch	33° 56.864'S 18° 51.705'E	Pears	50	4,871	16	0
Stellenbosch	33° 56.864'S 18° 51.705'E	Citrus	130	3,597	2	2
Stellenbosch	33° 56.864'S 18° 51.705'E	Apples	50	2,763	0	0
Stellenbosch	33° 56.864'S 18° 51.705'E	Apples	50	2,747	0	1
Stellenbosch	33° 56.864'S 18° 51.705'E	Plums	50	3,059	0	0
De Doorns	33° 28.480'S 19° 39.59'E	Table Grapes	50	1,733	0	0
Ceres	33° 55.03'S 18° 49.10'E	Plums	55	6,279	0	0
Ceres	33° 55.03'S 18° 49.10'E	Plums	55	6,451	0	0
Ceres	33° 55.03'S	Peaches	52	6,665	0	0

	18° 49.10'E					
Ceres	33° 55.03'S 18° 49.10'E	Peaches	50	5,437	3	0
Tulbagh	33° 16.35'S 19° 10.14'E	Pears	60	9,218	7	0
Tulbagh	33° 16.35'S 19° 10.14'E	Pears	10	1,160	0	0
Riebeeck Kasteel	33° 23.20'S 18° 54.00'E	Table Grapes	50	4,003	1	2
Robertson	33° 48.26'S 19° 53.01'E	Peaches	14	3,137	0	0