Potential Synergism between Entomopathogenic Fungi and Entomopathogenic Nematodes for the control of false codling moth (*Thaumatotibia leucotreta*)

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Abstract

False codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (FCM), is a major phytosanitary pest of citrus in South Africa. Sufficient control measures for the soil-dwelling life stages of FCM have yet to be identified and owing to restrictions on the use of insecticides, non-chemical control options have been investigated including the use of entomopathogenic fungi (EPF) and entomopathogenic nematodes (EPN). Laboratory and field trials on an indigenous EPF, *Metarhizium anisopliae* FCM Ar 23 B3, have shown that this isolate is capable of inducing mortality in FCM soil-dwelling life stages. Other agents that have been highlighted as potential controls for soil-dwelling FCM life stages are the EPN species *Steinernema yirgalemense* 157-C, *S. jeffreyense* J194 and *H. noenieputensis* 158-C. This study conducted laboratory bioassays to assess the virulence of these four control agents on fifth instar FCM, in 24-well plates. These results reaffirmed the virulence of the four microbial control agents at their recommended doses of 50 IJs (EPN) and 1×10^7 conidia/ml (EPF) against fifth instar FCM with 80 to 96% larval mortality recorded. The EPF isolate exhibited the lowest mortality whilst *S. yirgalemense* induced the greatest mortality. In addition, the lethal concentration (LC) values for each isolate were determined using dose response bioassays. These values were previously unknown for all EPN species and for the EPF isolate based on the methodology used in this study. The LC_{50} results in order from lowest to highest EPN IJ concentration requirements were 4.38 IJs (*S. yirgalemense*), 4.47 IJs (*S. jeffreyense*) and 7.11 IJs (*H. noenieputensis*). The EPF isolate exhibited an LC_{50} of 3.42×10^5 conidia/ml. Lastly, research has shown that the combination of two control agents may increase control of late instar lepidopteran and coleopteran larvae, through synergistic interactions. Thus, the interactions that occurred between the combination of these EPN species with the EPF isolate were determined. This study found that when all three EPN species were combined simultaneously and sequentially with the EPF isolate *M. anisopliae* FCM AR 23 B3, additive interactions took place with exception of the simultaneous application of *S. yirgalemense* and *H. noenieputensis*, with the EPF and *S. jeffreyense* applied 24 h post EPF application. For the former, a synergistic interaction was found, whilst for the latter two, an antagonistic interaction. Although no strongly synergistic interactions were observed, additive interactions have been shown to reach a synergistic level when certain parameters are changed. Moving forward, a uniform methodology for conducting EPF/EPN interaction experiments has been suggested. It has also been recommended that due to the additive interactions observed in this study, laboratory soil-bioassays and field trials should be carried out for all three EPN species in combination with the EPF isolate. This research will inevitably facilitate the constant knowledge into management strategies for the phytosanitary pest, FCM in South African citrus.

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List of Abbreviations

'	Minute(s)
''	Second(s)
%	Percentage
®	Registered Trademark
™	Trademark
°	Degree(s)
χ^2	Chi-squared
°C	Degree(s) Celsius
µl	Microlitre
Bt	*Bacillus thuringiensis*
Btj	*Bacillus thuringiensis* subspecies *japonensis* Buibui strain
CABI	Centre for Agriculture and Bioscience International
CBC	Conservation biological control
CE	Controlled environment
CGA	Citrus Growers' Association
cm^2	Square centimetre(s)
CRI	Citrus Research International
CrleGV	*Cryptophlebia leucotreta* granulovirus
DAFF	Department of Agriculture, Forestry and Fisheries
dH_2O	Distilled water
E	East
Ed(s).	Editor(s)

EPF	Entomopathogenic fungi
EPN	Entomopathogenic nematodes
ERH	Equilibrium relative humidity
et al.	*Et alia* (and others)
etc.	*Et cetera*
e.g.	*Exempli gratia*
EU	European Union
FCM	False codling moth
GST	Detoxifying enzymes glutathione S-transferase
GV	Granulovirus
h	Hour(s)
i.e.	*Id est*
IGR	Insect growth regulators
IJs	Infective juveniles
IPM	Integrated pest management
J1	Juvenile 1
J2	Juvenile 2
J3	Juvenile 3
J4	Juvenile 4
L	Litre
LC	Lethal concentration
LC_{50}	Median lethal concentration
MCA	Microbial control agents
MD	Mating disruption

ml	Millilitre
µm	Micrometre
MRL	Maximum chemical residue limit
NBTA	Nutrient bromothymol blue agar
NPV	Nucleopolyhedrovirus
pers. comm.	Personal communication
PO	Phenoloxidase
PPRI	Plant Protection Research Institute
RH	Relative Humidity
RPW	Red palm weevil
s. str	*Sensu stricto*
SDA	Sabouraud Dextrose Agar
S	South
Sp.	Species
Spp.	Species (plural)
TOD	Time of death
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
X	Magnification

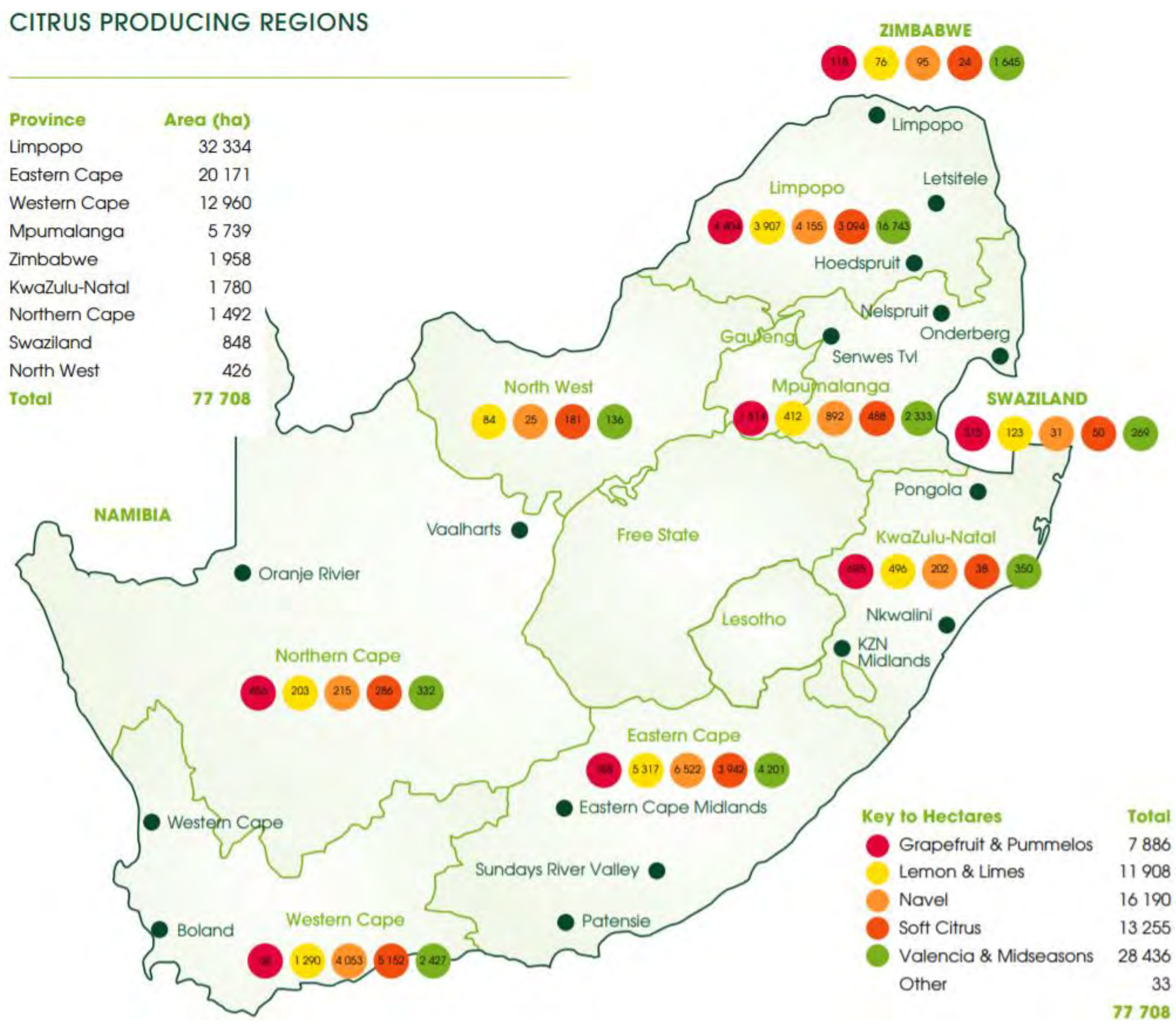
Chapter 1

GENERAL INTRODUCTION

1.1 CITRUS IN SOUTH AFRICA

1.1.1 Citrus Industry

The South African climate is highly favourable for fruit production, in particular *Citrus* (Sapindales: Rutaceae) (Department of Agriculture, Forestry and Fisheries (DAFF) 2017). South Africa is the 14th largest citrus producer in the world and the 2nd largest citrus exporter worldwide (CGA 2018a). In the 2017 citrus season 1.845 million tons of citrus, accounting for 76% of all production, was exported to foreign markets (CGA 2018b). Remaining citrus is either sold to local markets (6%) or processed into other citrus related products (18%) (CGA 2018a). Majority of South African citrus is exported to Europe (EU) (40%), South East Asia (17%) and the Middle East (17%) (CGA 2018a). Although citrus crops are grown across seven of the nine provinces, the key citrus producing areas are in Limpopo, Mpumalanga, the Eastern Cape and the Western Cape (Figure 1.1) (CGA 2018a). South Africa grows Valencia oranges, Navel oranges, lemons, grapefruit and soft citrus (CGA 2018a).



Province	Area (ha)
Limpopo	32 334
Eastern Cape	20 171
Western Cape	12 960
Mpumalanga	5 739
Zimbabwe	1 958
KwaZulu-Natal	1 780
Northern Cape	1 492
Swaziland	848
North West	426
Total	**77 708**

Key to Hectares	Total
🟥 Grapefruit & Pummelos	7 886
🟨 Lemon & Limes	11 908
🟧 Navel	16 190
🟧 Soft Citrus	13 255
🟩 Valencia & Midseasons	28 436
Other	33
	77 708

Figure 1.1. Citrus producing regions showing the citrus production for those areas including the citrus types grown in those areas in South Africa and Swaziland (now Eswatini) (Taken from CGA 2018b).

1.1.2 Citrus Pests

The citrus industry in South Africa currently has close to 100 insect pests with ten different insect species that are classified as economically important pests (Smith & Peña 2002; Grout & Moore 2015; De Meyer et al. 2016; Manrakhan et al. 2018). These pests are: the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), Natal fruit fly, *Ceratitis rosa* (Karsch) (Diptera: Tephritidae, Red scale, *Aonidiella aurantii* (Maskell) (Hemiptera: Diapsidae), Citrus psylla, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), Citrus thrips, *Scirtothrips aurantii* (Faure) (Thysanoptera: Thripidae), Citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae), Oleander mealybug, *Paracoccus burnerae* (Brain) (Hemiptera: Pseudococcidae), Long-tailed mealybug, *Pseudococcus longispinus* (Targioni-Tozzetti) (Hemiptera: Pseudococcidae) (although there are four other mealybug species that occur on citrus in South Africa) (Smith & Peña 2002; Grout & Moore 2015; De Meyer et al. 2016; Manrakhan et al. 2018) and false codling moth (FCM), *Thaumatotibia leucotreta* Meyrick (1912) (Lepidoptera: Tortricidae). The last mentioned, FCM, is the most important pest in South Africa due to its phytosanitary status (Venette et al. 2003; Moore 2021). FCM originated in sub-Saharan Africa (Stibick 2010). It has a wide host range of over 100 plant species, which comprises of both non-cultivated and cultivated plants (Venette et al. 2003; Kirkman & Moore 2007; CABI 2021) including citrus. In South Africa, Navel oranges and mandarin varieties are the most susceptible cultivar for FCM oviposition and infestation, due to the chemical compounds within these varieties (Love et al. 2014). Whereas grapefruit and soft citrus have been found to be the least susceptible cultivars to FCM infestation (Gunn 1921), and lemons are not attacked by this pest (Moore et al. 2015a).

1.2. FALSE CODLING MOTH (FCM)

1.2.1 Classification

False codling moth (FCM), *Thaumatotibia leucotreta* Meyrick (1912) (Lepidoptera: Tortricidae), is a native insect to Southern Africa. FCM was first discovered in Natal (now KwaZulu-Natal) in 1901 by Fuller (Catling & Aschenborn 1978). Originally, FCM was classified under the genus *Carpocapsa* (Schwartz 1981). However, in 1912 Meyrick changed the classification to *Argyroploce leucotreta* (Eucosmidae: Olethreutidae) (Brown 2005). In 1958 the classification was then further revised by Clarke, placing FCM into the genus *Cryptophlebia* (Newton 1998). In 1999 the species *leucotreta* was then reclassified and placed into a different genus, *Thaumatotibia*, which remains the current classification for FCM (Newton 1998; van den Berg 2001; Venette et al. 2003).

1.2.2 Life Cycle

FCM oviposition occurs on the rind of the fruit during dusk (17:00 and 23:00) and the eggs take between 4 to 12 days to develop (Daiber 1979a, 1980) depending on temperature. Once the eggs have hatched the 1.5 mm long first instar larvae (neonates) tunnel into the fruit and undergo development, which takes 25 to 67 days, depending on the quality and cultivar of the fruit, as well as temperature (Daiber 1979b; Newton 1998, Love et al. 2014). There are five larval instars and the larvae remain in the fruit, consuming the fruit pith until they reach fifth instar. Fifth instars are approximately 12 to 15 mm in length and have changed from a cream-white to a dark pink colour (Figure 1.2) (Daiber 1979c, Newton 1998; van den Berg 2001; Grout & Moore 2015).

The fifth instars will leave the fruit once they near pupation and drop into the soil below the host tree, which initiates the subterranean FCM life stage (Daiber 1979b). The larvae will form cocoons that comprise of self-spun silk, soil and debris, within the upper most layers of the soil profile (Newton 1998; van den Berg 2001; Georgala 1969; Grout & Moore 2015; Love 2015). Pupation lasts for 21 to 40 days until the adults eclose (Newton 1998). FCM adults have a wingspan of 16 to 20 mm and have mottled brown-grey forewings and paler hind-wings (Figure 1.2) (van den Berg 2001). The lifespan of FCM adults is 1 to 3 weeks. During this time the adults do not feed, but water uptake is essential (Catling & Aschenborn 1974). Mating will occur shortly after eclosion and a 1:1 sex ratio is typically observed in the field (Newton 1998; van den Berg 2001). The variation in each developmental stage and overall duration of the FCM lifecycle is influenced by temperature and food quality (Stibick 2010). FCM does not undergo diapause and is therefore a continual pest with 5 to 6 overlapping generations (Terblanche et al. 2014).

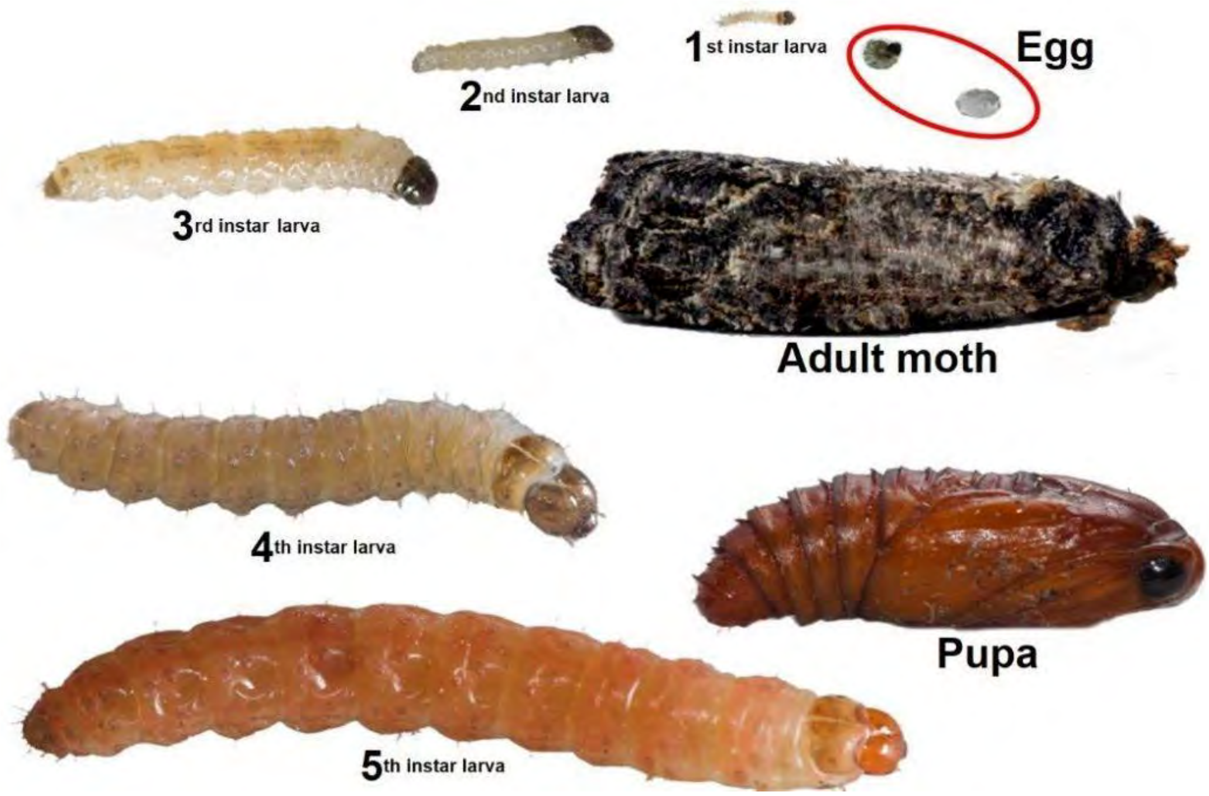


Figure 1.2. False codling moth life cycle (Taken from Madumbi 2019).

1.2.3 Economic importance of FCM

During the FCM life cycle the citrus fruit is damaged in two ways: direct damage (pre-harvest) and indirect damage (post-harvest). When the neonates tunnel into the fruit, they directly damage the fruit, resulting in fruit decay, premature ripening and early detachment of the fruit from the trees (Newton 1998). The holes in the fruit caused by the neonate tunnels result in indirect damage by providing a point of entry for plant pathogens like blue mould (*Penicillium italicum* Wehmer) and green mould (*P. digitatum* Sacc.) (Newton 1988; Newton 1998). The pre- and post-harvest damage caused by FCM results in financial losses to farmers and the industry at large, with an (outdated) estimated ZAR 100 million lost as a result of FCM infestations (Kirkman & Moore 2007).

Due to the damage caused by FCM, the value of citrus crops and its endemism in Africa and localised in Israel (Wysoki 1986), FCM is classified as a phytosanitary pest (Moore 2021). The FCM phytosanitary restrictions involve a no entry policy within the export markets of the United States of America, Europe and South Korea and China (Venette et al. 2003; Grout &

Moore 2015; Moore 2021). If a single live FCM larva is detected in fruit destined for these markets with phytosanitary restrictions, the entire shipment will be rejected (Moore 2012). These phytosanitary restrictions on FCM can be detrimental to the citrus economy in South Africa, considering the bulk of South African citrus production, as previously stated, is exported (CGA 2018b). Therefore, managing and reducing FCM levels within South African orchards is imperative for the citrus economy (Grout & Moore 2015).

1.2.4 FCM Control

Due to the negative effects that FCM infestations have on the South African citrus industry, numerous control strategies have been implemented in South Africa, to manage this key pest species. FCM control involves preharvest and postharvest control strategies. Moore (2019, 2021) provides an in-depth account of the current control measures for FCM in South Africa, specifically within the citrus industry. A brief overview of the preharvest control strategies will be discussed, with focus on the role of biological control, entomopathogenic fungi and entomopathogenic nematodes.

1.2.4.1 Chemical control

Agricultural crop pests were initially controlled by synthetic insecticides, such as pyrethroids, organophosphates and insect growth regulators (IGR) (Moore & Hattingh 2012). The most recent guidelines for chemical control for FCM in South Africa are outlined in the Citrus Research International (CRI) FCM management guidelines (Moore 2019). From these guidelines eight commercially available chemical insecticides are available for FCM control in South Africa (Moore & Hattingh 2012; Coombes 2015; Moore 2019). These being: Alystin® (IGR) and Nomolt® (IGR), Runner® (and other generics) (Methoxyfenozide), Cypermethrin® (pyrethroid), Meothrin® (pyrethroid), Delegate®, Coragen® and Warlock®. Chemical insecticides are used to target the egg and larval FCM life stages. Although certain insecticides have shown to be compatible with IPM programmes, there are several disadvantages which has resulted in their restricted use in both local and foreign markets. Chemical insecticides can be detrimental to non-target pests as well as lead to secondary pest outbreaks due to their broad-spectrum abilities (Michaud & Grant 2003). Insecticides can also be misused, including overuse, which can result in acquired resistance to these chemicals in pest populations, rendering them useless (Aidley 1976). In the San Joaquin Valley, USA, insecticide resistance was observed amongst populations of citrus thrips (Morse & Brawner 1986) and California red scale and yellow scale (Grafton-Cardwell et al. 2001) due to heavy use of organophosphate and carbamate insecticides in the 1950s – 1990s in citrus orchards. In the 1970s, California red scale also developed resistance to organophosphates in South Africa (Nel et al. 1979). Insecticide resistance has also been documented in FCM, however, this was only shown after about seven years of frequent usage (Hofmeyr & Pringle 1998). Insecticides can also bioaccumulate within the agroecosystem and surrounding environment, which can negatively affect other wildlife, as these chemicals can be toxic (Aidley 1976). However, insecticides currently registered are less toxic than their predecessors. Some toxic insecticides like Meothrin® and Cypermethrin® may only be used once for FCM per growing season and have strict regulations preventing their use leading up to the harvesting season (Coombes 2015). Export markets such as the UK, EU and USA have also imposed restrictions on insecticides by reducing the maximum chemical residue limit on fruit (MRL) (Grout & Stephen 2005; Kirkman 2007). These restrictions have been put in place due to the negative environmental impacts that insecticides pose and, more importantly, the food safety concerns related to human health upon exposure to these chemicals. Human exposure to insecticides can occur through the direct spraying of these chemicals by farm workers and through contaminated food (Pekas 2011). Essential oils obtained from citrus are prime examples of how humans can be exposed indirectly to insecticides, because these oils are used in the food, cosmetic and pharmaceutical industries (Pekas 2011).

Due to the negative effects of chemical insecticides within the citrus industry, a broader and more sustainable control approach has been implemented, through integrated pest management (IPM) (Moore & Hattingh 2012). IPM makes use of chemical, cultural and biological control to bring about a reduction in pest populations within the South African citrus industry. Citrus Research International (CRI) has implemented an IPM programme for phytosanitary pests like FCM in the South African Citrus Industry (Moore & Hattingh 2012). The combined control measures used in the citrus FCM IPM programme have been incredibly successful and can result in a 97% reduction in FCM infestation when implemented correctly (Smith & Peña 2002; Moore et al. 2015b; Moore et al. 2017).

1.2.4.2 Orchard Sanitation

Cultural control is one aspect of the systems approach within FCM IPM and is comprised of mandatory manual and mechanical techniques to control FCM. Orchard sanitation aims to reduce the level of pests and pathogens within the orchards by manually removing any diseased plant material. Orchard sanitation practices also involve the monitoring of water and soil within the orchard and more importantly the monitoring of the citrus trees during their different growth stages (Food and Fertilizer Technology Center 2003). This process aids in preventing the loss of tree productivity or death (Food and Fertilizer Technology Center 2003). For the management of plant pathogens, key strategies involve the removal of fallen leaves and twigs, pruning diseased branches and ensuring the soil surface is covered to reduce fungal infections within the root systems (Food and Fertilizer Technology Center 2003). For insect pests such as FCM, orchard sanitation practices involve the removal of infected fruit both on the tree and on the ground (Hill 1983). If orchard sanitation is carried out weekly, Moore & Kirkman (2008) found that a 75% reduction in fruit infestation can result (Moore & Hattingh 2016). Orchard sanitation practices should be carried out regularly to induce a significant reduction of pathogen and pest populations/outbreaks (Hill 1983).

1.2.4.3 Sterile insect technique

Another method of manually controlling pest populations is through the sterile insect technique (SIT), which is used to inhibit FCM adult insect reproduction by releasing sterile males into the orchards (Stotter et al. 2014). This will result in a reduction in the number of viable progenies, therefore reducing the population levels within the orchards (Stotter et al. 2014). When SIT has been used commercially in an integrated programme since 2007, it has resulted in a reduction in fruit infestation (96%), moth catches (99%) and export rejections (89%) (Barnes et al. 2015).

1.2.4.4 Mating disruption

Mating disruption (MD) control strategies for FCM aim to reduce the number of viable eggs, and therefore decrease the population size of FCM infestations within citrus orchards (Moore 2019). MD is brought about through disorientating male moths because the FCM females are competing with the synthetic female sex pheromones (Moore 2019). Currently Checkmate® FCM-F, Isomate, Splat FCM™ and X-Mate™ FCM are four mating disruption products available for FCM control (Moore & Hattingh 2012; Moore 2019). However, this type of FCM control is only beneficial in low FCM pressure areas (Moore & Hattingh 2012). An in-depth description of these products and the optimal times to apply them are outlined in Moore (2019).

1.2.4.5 Biological control

Biological control utilises the natural enemies of the target pest to obtain successful control of pest populations (Murdoch et al. 1985). Biological control agents are characterised as host-specific, having a life cycle that synchronises with the pest, can increase in numbers when the host population increases, have a high searching ability and can remain within the pest population at low densities (i.e. persistent) (Murdoch et al. 1985). There are three categories of biological control: classical control, conservation control and augmentative control (Barbosa 1998; McCrevy 2008; Hoy 2008a, b).

Previous research has revealed that there are numerous predators and parasitoids of FCM that have been found in sub-Saharan Africa (Moore 2002). Moore (2002) provides a summary of the earlier research into potential FCM control agents, which includes mostly Hymenopteran and Dipteran parasitoids and predators. Although several insect species have been identified, the most successful insect biological control agent for FCM, is the egg parasitoid wasp, *Trichogrammatoidea cryptophlebiae* Nagaraja (Hymenoptera: Trichogrammatidae), which is found naturally in citrus orchards (Figure 1.3) (Moore & Hattingh 2016). *Trichogrammatoidea cryptophlebia* can also be mass reared using *in vivo* insect cultures (Moore & Hattingh 2012), which allows for augmentation of the wasp into citrus orchards, resulting in up to 60% reduction in FCM infestation (Newton & Odendaal 1990; Moore & Hattingh 2004, 2012).

Figure 1.3. *Trichogrammatoidea cryptophlebiae* wasp emerging from parasitised FCM egg (Taken from BioResources 2016).

The remaining biological control agents for FCM fall within the microbial control agents (MCA) category and include fungi, viruses, bacteria and nematodes (Lacey 2017). There are several advantages to MCA such as: 1) they are host specific and non-target organisms such as pollinators, natural enemies and earthworms are not harmed, 2) they may persist within the environment and therefore offer long term control, 3) they are classified as 'low-risk' controls and do not have maximum residue limitations (MRL), 4) they can easily be mass produced in artificial media (or *in vivo* in the case of viruses) and when formulated and stored correctly, they have a long shelf-life, 5) they can be applied with conventional spray equipment, 6) genetic modification is possible and 7) they are useful for resistance management in IPM programmes, because they provide an alternative control method to chemical control (Feng et al. 1994; Thomas et al. 1995; Price et al. 1997; Goettel et al. 2001; Lomer et al. 2001; Pu et al. 2005; Zimmerman 2007a, b; Farenhorst et al. 2009, 2010; Chandler et al. 2011; Garrido-Jurado et al. 2011; Lacey 2017).

However, there are some disadvantages when using MCA, these being: 1) initial production costs are high, 2) sensitivity to environmental conditions (however, formulation can mitigate these factors), 3) they are most effective when used in IPM programmes because they cannot always provide standalone control, 4) they have a shelf-life, which is dependent on formulation, 5) slower mortality rates compared to insecticides and 6) resistance can occur, although this is much reduced when compared to chemical products (e.g. *Cydia pomonella* to codling moth GV) (Lacey 2017).

Three types of MCA are used in FCM IPM programmes in South Africa. FCM virus control agents fall within the family Baculoviridae. There are two types of baculoviruses: 1) Granuloviruses (GV) (Betabaculovirus) and 2) Nucleopolyhedrovirus (NPV) (Alphabaculovirus, Gammabaculovirus and Deltabaculovirus). In South Africa one entomopathogenic virus-based product has been developed thus far, Cryptogran® (River Bioscience, South Africa) (Moore 2012; Barnes et al. 2015; Moore et al. 2015b; Hatting et al. 2019). Cryptogran® consists of the virus Cryptophlebia leucotreta granulovirus (CrleGV-SA), a native pathogen found in South Africa. This pathogen has been used for over 15 years in South Africa and has shown to reduce FCM infestations up to 92% (Moore et al. 2015b). Cryptex and Gratham also incorporate CrleGV as their active ingredient, albeit a different strain (Hatting et al. 2019). Both of these registered products are developed in Switzerland (S.D. Moore, pers. comm.).

Currently two species of EPF have been investigated for the control of FCM, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Coombes et al. 2016). To date *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) was the only EPN that had been registered for controlling FCM in the South African citrus industry (Hatting et al. 2019). However, it has since been deregistered due to issues surrounding its alien isolate status (S.D. Moore, pers. comm.). EPF and EPN play a crucial role in combating the soil-dwelling life stages of FCM, which still requires further research and product development.

1.3 ENTOMOPATHOGENIC FUNGI

1.3.1 Classification

Entomopathogenic fungi (EPF) belong to the fungal kingdom and are characterised by their specific parasitic relationship with insects and other terrestrial arthropods, using these organisms as nutrient hosts. There are an estimated 700 EPF species, which accounts for less than 1% of the described fungal species (Roberts & Humber 1981; McLaughlin et al. 2009; Goettel et al. 2010; Vega et al. 2012). The phenomenon of entomopathogenicity is found throughout the fungal kingdom and is not restricted to a single phylum/subphylum (Roberts & Humber 1981; Chandler 2017). EPF are found within four main phyla: Entomophthoromycota, Blastocladiomycota, Microsporidia and Ascomycota (Humber 2008). Microsporidia are ubiquitous, obligate intracellular pathogens of both invertebrates and vertebrates and were originally thought to be protozoans and classified within the phylum Apicomplexa (Schwartz 1998; Corradi 2015). However, in some studies this group has been considered as EPF due to the belief that microsporidians form part of the earliest lineages of the fungal kingdom (Hibbett et al. 2007; Hirt et al. 1999; James et al. 2006). The Phylum Blastocladiomycota also contains a small group of insect pathogens (Mora et al. 2017). The Phylum Entomophthoromycota contains a large number of EPF species, characterised as obligate entomopathogens that are highly specialised parasites (Humber 2008). Although the Phylum Ascomycota does not have the greatest number of EPF species as Entomophthoromyctoa, it is the largest group within the fungal kingdom, and falls within the subkingdom Dikarya, along with the Phylum Basidiomycota (Araújo & Hughes 2016).

Ascomycota are characterised as obligate biotrophic parasites that colonise the insect cuticle resulting in disease or death of the host (Weir & Blackwell 2001). This phylum is divided into three subphyla; Saccharomycotina, Taphrinomycotina and Pezizomycotina (Mora et al. 2017). Taphrinomycotina do not contain EPF species, instead they are vertebrate parasites or plant saprophytes (Mora et al. 2017). Saccharomycotina (ascomycete yeasts) do have some level of association with insects, whereby insects are used as modes of dispersal and the fungi provide their hosts with resources such as vitamins and enzymes (Vega & Dowd 2005). Pezizomycotina contain the EPF species within the division Ascomycota and are the most complex subphyla due to their anamorphic (asexual) and teleomorphic (sexual) life stages (Mora et al. 2017). Pezizomycotina is further divided into four classes Tubeuflaceae, Eurotiomycetes, Laboulbeniomycetes and Sordariomycetes. Tubeufiaceae contain a variety of associations/feeding strategies including saprobic and parasitic forms (Kodsueb et al. 2006). Eurotiomycetes contains the order Onygenales, which is one of the key EPF groups within Ascomycetes, specifically the genus *Ascosphaera*, which are obligate honeybee parasites (Sosa-Gómez et al. 2010).

The Sordariomycetes class contains the order Hypocreales, which contains the greatest number of well-known EPF in the phylum Ascomycota. Clavicipitaceae was originally the main family within Hypocreales however, it has now been split into three monophyletic families: Clavicipitaceae, Cordyicipitaceae and Ophiocordycipitaceae (Chandler 2017; Mora et al. 2017). Well-known EPF species are found within these three families, such as *Metarhizium* and *Regiocrella* (Clavicipitaceae) (Sung et al. 2007), *Cordyceps* species, including the asexual species *Beauveria, Lecanicillium* and *Isaria* (Cordycipitaceae) (Mora et al. 2017) and ant and termite pathogens (Ophiocordycipitaceae) (Sung et al. 2007).

1.3.2 General Biology

The phylum Ascomycota contains fungal species that have both anamorphic (asexual) and telomorphic (sexual) life cycles (Figure 1.4) (Shah & Pell 2003; Inglis et al. 2001). Anamorphic fungi produce conidia through asexual reproduction on the hyphae (Chandler 2017), unlike the telomorphic sexual reproductive life cycle illustrated in Figure 1.4. The two life cycles were originally split into two groups Ascomycota (sexual state) and Deuteromycota (no sexual state) however, molecular studies revealed that a fungal species contained both sexual and asexual states which should be recognised as a single species (Shah & Pell 2003; Inglis et al. 2001).

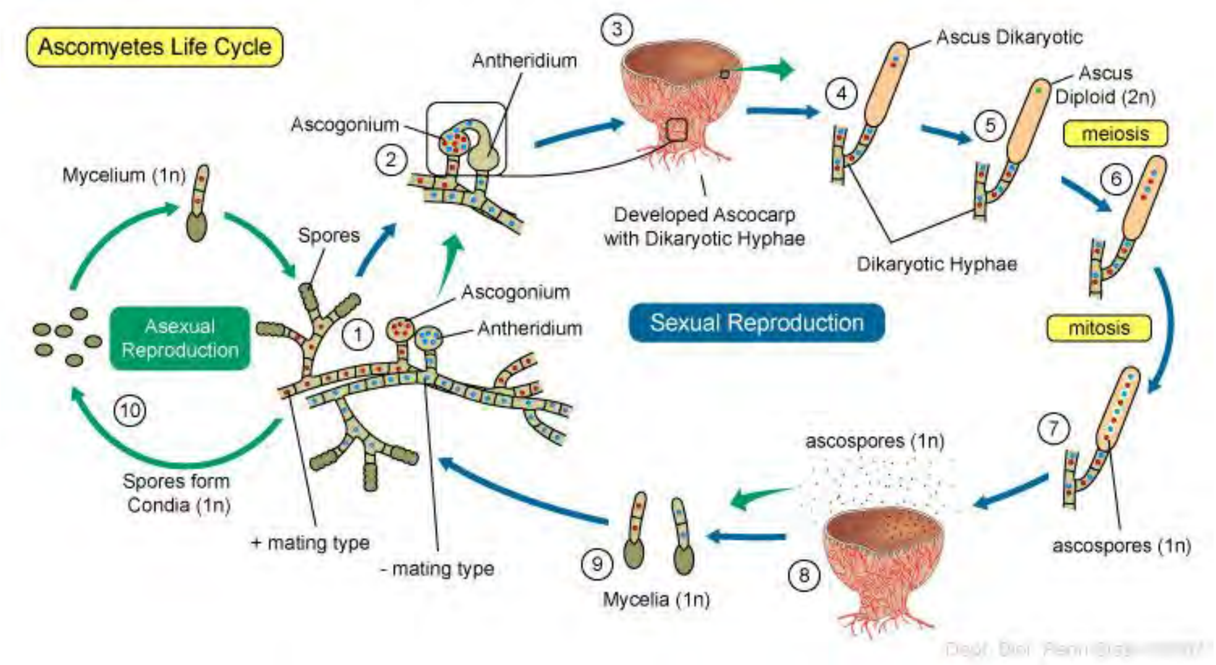


Figure 1.4. Asexual and sexual life cycle of Ascomycota fungi (Taken from Department of Biology Penn State University 2002).

The remainder of the EPF section in this thesis focuses on asexual hypocrealean fungi as MCA, which exhibit both parasitic and saprophytic behaviours. The saprophytic phase is believed to have originated from EPF early saprophytic ancestors which resided in soil and leaf litter (Humber 1984; Samson & Evans 1988; Evans 1989; Spatafora & Blackwell 1993). EPF are found within the soil profile, which protects fungal mycelia and spores from UV radiation and temperature fluctuations as well as ensuring water availability, necessary for spore germination (Vega et al. 2009). The parasitic phase involves the relationship between arthropods (hosts) and EPF (pathogen), driving the evolutionary “arms-race” which influences EPF virulence and arthropod host defences (Pedrini et al. 2015). In order for EPF to encounter their insect hosts within the soil environment, it is important for EPF to synchronise their life cycles with their insect hosts to ensure contact and infection of the host is achieved (Shah & Pell 2003). Synchronising of the host-EPF life cycles depends on the abundance and presence of the host in the natural environment and the presence of alternative nutrient sources for the EPF (Chandler 2017). Certain EPF species have endophytic abilities, allowing them to inhabit plant tissues. In order for infection of the insect host to occur the EPF needs to be in the parasitic conidial form and not in the endophytic form (Backman & Sikora 2008; Wilson 1995). Other factors that impact host/pathogen interactions are the fungal host range (specialist/generalist), fungal germination and infection rates and optimal temperatures for infection (Chandler 2017; Sierotzki et al. 2000; Pell et al. 2001; Shaw et al. 2002).

1.3.4 Generalised Life Cycle: Asexual Hypocrealean EPF

Hyprocrealean fungi switch between two cycles, the saprophytic and parasitic (insect host) cycles. Between these two cycles a plant association period may also occur where the fungi is able to survive on organic non-host substrate within the environment (Figure 1.5). Infection of the host by entomopathogenic fungi begins when the spore (conidia) encounters and adheres to the host cuticle (Chandler 2017). Hypocrealean conidia are small (< 8μm) hydrophobic spores, and a high number of conidia is required to induce successful infection of the host (Chandler 2017, Hesketh et al. 2010). The conidia contain proteins on their cell walls which aid in adhesion to the hosts cuticle, these proteins include phospholipases, superoxide dismutases, aspartyl proteases and adhesins (De Groot et al. 2013; Pitarch et al. 2008). For attachment to occur a specific minimum equilibrium relative humidity (ERH) of 93%, an optimal temperature and oxygen levels are required at the site of infection to induce spore germination (Andersen et al. 2006). The increase in water and oxygen causes the spore to swell due to hydration, which is followed by the production of a thin-walled appressorium (germ tube) (Chandler 2017). The germ tube then penetrates the host's epicuticle through a combination of concentrated physical energy and lytic enzymatic activity (Hajek & St. Leger 1994). The production of tube-like hyphae then makes use of the hosts epicuticular waxes and lipids for growth and nutrition by secreting degradative enzymes into the environment and reabsorbing the broken-down products into the fungal cells (Chandler 2017). The procuticle, a significant physical and chemical barrier against infection, is then penetrated by the hyphal tips, which grow through aid from enzymatic degradation (Chandler 2017). The procuticle contains chitin layers that are embedded within a protein matrix (St Leger et al. 1987). The hyphae continue to penetrate the host cuticle, until entering the haemocoel, where two host immune responses are triggered, firstly the encapsulation of invading fungal cells and secondly, the melanisation of fungal tissues (Chandler 2017). However, Hypocrealean fungi have defences that allow them to evade the hosts immune defences. The production of blastospores, yeast-like hyphal bodies, ensure rapid dispersal of fungal bodies within the haemolymph and prevent detection from the host's pathogen recognition molecules (Jing et al. 2010). A reduction in feeding or flight activity as well as necrotic spots are signs of fungal infection

(Moore et al. 1992; Seyoum et al. 1994; Roy et al. 2006). Entomopathogenic-induced host death occurs due to the physical damage and loss of normal functioning, brought about when the fungal biomass replaces the host's soft tissue and other factors including the negative effects of fungal metabolites (e.g. toxins), host starvation and water loss (Chandler 2017). The time of death (TOD) varies between 3 – 20 days after infection, depending on the fungal species and the physical state of the host (Hesketh et al. 2010).

Following host death, the fungus will grow out of the host, through the cuticle and produce spores (conidia) which are used to transmit the fungus to a new host (Chandler 2017). The process of spore production requires favourable external environmental conditions (Arthurs & Thomas 2001). In the case of unfavourable conditions, the host cadaver will remain in a mummy-like form until favourable conditions occur and spore production can take place (Chandler 2017). The production of spores from the host cadaver occurs over a short period of time and spores can persist within the environment for long periods of time, which can result in a reservoir of spores collected within the soil (Hesketh et al. 2010; Meyling & Eilenberg 2007; Meyling et al. 2011). Once the conidia are present within the environment, new hosts can contract fungal conidia when they encounter a sporulating cadaver or contact spore reservoirs (Hesketh et al. 2010; Kreutz et al. 2004). Host mating can also act as a mode of transmission (Kaaya & Okech 1990). The small spore size increases the distribution ability of the fungal species, because water splash or wind can facilitate the indirect dispersal of these spores (Chandler 2017). The success of EPF infections and host death can be high. Depending on the species and virulence of each isolate, mortality rates of 100% are not uncommon due to the rapid intra-host growth rates and transmission to new hosts (Chandler 2017).

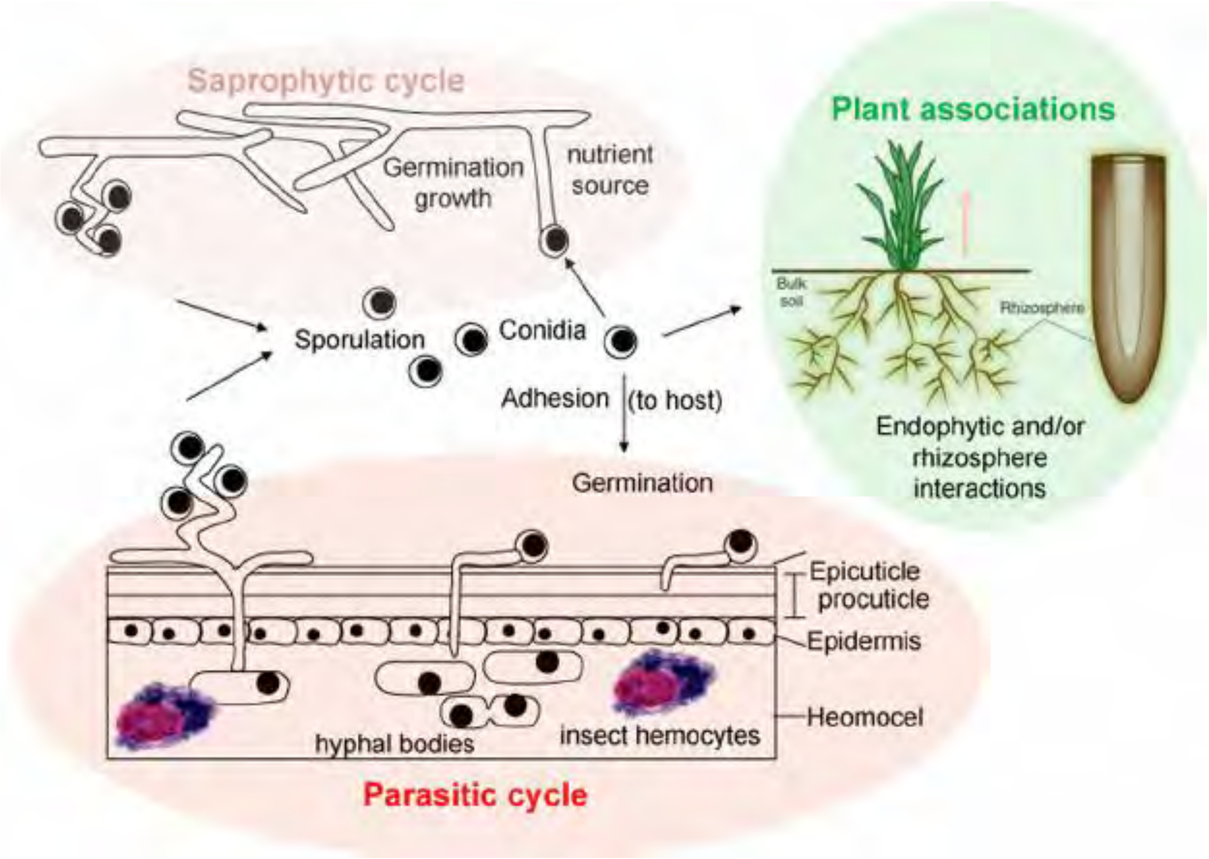


Figure 1.5. Generalised life cycles of hypocrealean fungi: Saprophytic and parasitic cycle, including the plant association stage (Taken from Ortiz-Urquiza et al. 2014).

1.3.5 Use as MCA

EPF make up an important part of terrestrial ecosystems and natural enemy complexes, responsible for regulating insects in the soil (Meyling & Eilenberg 2007; Quesada-Moraga et al. 2007). However, for a number of years EPF along with other MCA were often overlooked as potential control methods for combating agricultural pests (Chandler 2017). This is attributed to the lack of understanding surrounding EPF ecology, agricultural practices, and the success of chemical insecticides. Nevertheless, due to the negative effects of chemical pesticides (section 1.2.4.1), a new market has opened for biopesticides (Chandler 2017). Currently, limited EPF products are available for controlling agricultural pests with half the registered fungal biopesticide products originating from Central and South America, 20% from North America, 12% from Eurasia and only 3% from Africa (Faria & Wraight 2007). Most of the available commercial EPF are anamorphic Hypocreales species, such as *Beauveria bassiana, Metarhizium* spp., *Isaria fumosorosea* and *Lecanicillium* spp. (Chandler 2017).

The investigation into new EPF products is increasing as agricultural practices move towards safer control methods and implement IPM programmes (Marrone 2007). Successful fungal MCA products need to consider the following factors, to ensure sufficient control is achieved: isolate virulence, phylogeny, phenotypes, environmental hardiness (susceptibility to UV radiation and temperature tolerance) and host specificity (Chandler 2017). It is also important for potential fungal isolates to be selected from indigenous EPF populations because releasing alien EPF species could pose problems for indigenous ecosystem dynamics. Utilising indigenous EPF species forms part of conservation biological control (CBC) which alters farming practices to protect or maintain the natural enemies present within the environment, which can aid in the suppression of the pest population (Meyling & Eilenberg 2007).

In a citrus context, orchards have been shown to support strong microbial communities, and have exhibited positive results when using MCA (Lacey et al. 2008). A good example of this is the successful field trials exhibited in Coombes et al. (2016) which alluded to the great potential of indigenous EPF as control agents for FCM in South African citrus. Due to the success of other MCA and the availability of EPF in soil communities using EPF as MCA is promising. However, the majority of EPF products are used in the horticultural industry primarily within greenhouses (fruit and vegetables) and ornamental crops (Chandler 2017). This is because EPF persist longer in green house environments because they eliminate the harmful factors associated with the natural environment. Overcoming these factors is important when producing new EPF MCA.

1.3.6 EPF in South African citrus

Microbial control agents such as EPF have shown to be successful in controlling numerous orchard pests (Lacey & Shapiro-Ilan 2003, 2008; Alves et al. 2005). Currently only three EPF products are registered in South Africa, BroadBand™ (BASF, South Africa), Eco-Bb® (Plant Health Products, South Africa), both utilising *B. bassiana* strains, and Real Metarhizium 69 (Real IPM, Nairobi, Kenya), produced from a *M. anisopliae* strain (Goble et al. 2011; Coombes et al. 2013). Therefore, bioprospecting for new local EPF found within citrus orchard soils has been investigated in South Africa, particularly in the Eastern Cape Province, with the hope of producing more EPF products in the future. A study conducted by Goble et al. (2010) found 62 potential EPF isolates, with the two most common isolates being *Beauveria bassiana* (15.63%) and *Metarhizium anisopliae* (3.82%). These two genera are known as common inhabitants of soil in temperate regions, including agroecosystems (Chandler et al. 1997; Keller at el. 2003) and have been found to infect FCM and fruit fly species (Coombes et al. 2016; Goble et al. 2011; Dimbi et al. 2003; Lacey & Shapiro-Ilan 2003; Begemann 2008).

From the results obtained by Goble et al. (2010, 2011), three promising fungal isolates were selected for the control of FCM in South Africa, *M. anisopliae sensu lato* Metchnikoff (Sorokin) G 11 3 L6, *M. anisopliae sensu stricto* (*s. str*) FCM Ar 23 B3 and *B. bassiana s. str* Balsamo (Vuillemin) G Ar 17 B3 and required further testing (Coombes et al. 2013). The three isolates were found to successfully induce mortality in soil-dwelling life stages, wandering fifth instar larvae (Coombes et al. 2013, 2016). Further information was required to determine whether these isolates would make suitable control agents. The persistence and field efficacy of the three isolates was investigated and it was determined that only *M. anisopliae* FCM Ar 23 B3 and *B. bassiana* G Ar 17 B3, would be suitable control agents (Coombes et al. 2016). Both isolates were shown to persist within the environment for at least five months after application and were able to reduce FCM infestation by 28 and 82% (Coombes et al. 2016). However, environmental factors (section 1.3.5.1) were suggested to negatively impact field results (Coombes et al. 2016). Persistence within the environment is key for EPF when controlling FCM, due to the year-round presence of this pest, as there is no diapause (section 1.2.2). Therefore, further investigation was required to determine how resilient the fungal isolates were to environmental factors. Acheampong et al. (2020a, b) investigated how the UV sensitivity, humidity requirements and temperature tolerance effected the efficacy of seven EPF isolates, including the two identified above.

1.3.6.1 *Metarhizium anisopliae* FCM Ar 23 B3

Based on the findings by Goble et al. (2011), Coombes et al. (2013, 2016) and Acheampong et al. (2020a, b), the isolate *M. anisopliae* FCM Ar 23 B3 was selected for further testing and is the focus EPF isolate of this thesis.

1.4 ENTOMOPATHOGENIC NEMATODES

1.4.1 Classification

Nematodes (Phylum: Nematoda) are free living or parasitic non-segmented round worms (Shapiro-Ilan et al. 2017). Entomopathogenic nematodes (EPN) and parasitic nematodes evolved from free-living species (Figure 1.6) (Dillman et al. 2012). Parasitic nematodes and entomopathogenic nematodes (EPN) differ from each other. Parasitic nematodes feed on the host while the insect is still alive, whilst entomopathogenic nematodes rapidly induce host death and then begin feeding (Dillman et al. 2012; Abate et al. 2018). EPN, the focus of this study, belong to two families: Steinernematidae (Chitwood & Chitwood 1937) and Heterorhabditidae (Poinar 1976) (Stock & Goodrich-Blair 2008; Shapiro-Ilan et al. 2017). Steinernematidae contains the genus *Steinernema* along with one other genus and Heterorhabditidae contains the genus *Heterorhabditis* (Stock & Goodrich-Blair 2008). Research into EPN pathogenicity and biology only began in 1955 with Jaroslav Weiser's description of *Neoaplectana carpocapsae* from codling moth larvae (Poinar & Grewal 2012). Using early techniques (morphological and morphometric comparisons and crossbreeding tests), three *Heterorhabditis* and 10 *Steinernema* species had being identified by the late 1980s (Kaya & Gaugler 1993). However, with the addition of DNA analysis, and an increase in EPN research, the number of species belonging to *Steinernema* and *Heterorhabditis* have increased to 100 and 16, respectively (Hunt 2016; Nguyen et al. 2007; Stock & Goodrich Blair 2008; Hunt & Subbotin 2016; Abate et al. 2018). Species from the *Steinernema* and *Heterorhabditis* genera will be the focus of this thesis.

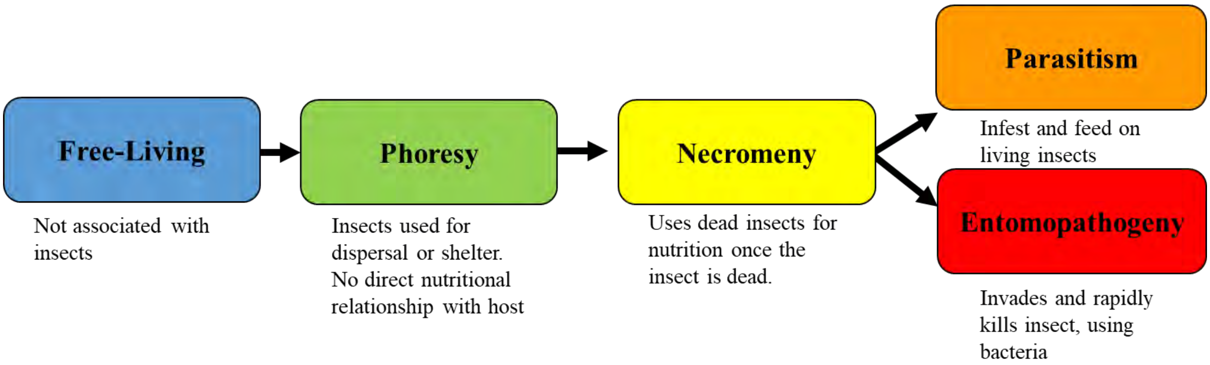


Figure 1.6. The evolution of nematode associations with insects, initially starting from non-associated free-living species to entomopathogenic nematodes (Adapted from Dillman et al. 2012).

1.4.2 General Biology

EPN have a mutualistic relationship with bacteria, which is responsible for inducing disease and death of the insect host. The EPN then consume both the insect and bacteria as nutrient resources (Dillman et al. 2012). *Steinernema* EPN are associated with bacteria in the genus *Xenorhabdus*, whilst *Heterorhabditis* EPN are associated with bacteria in the genus *Photorhabdus* (Poinar 1990; Lewis & Clarke 2012; Abate et al. 2018). *Xenorhabdus* and *Photorhabdus* belong to the family Enterobacteriaceae and are gram-negative rod-shaped bacteria (Stock & Goodrich-Blair 2008). Each EPN species has a mutualistic association with one bacterial symbiont species, although one bacterial species may be associated with more than one nematode species (Fischer-le Saux et al. 1999; Boemare 2002; Stock & Goodrich-Blair 2008). The mutualistic relationship between the nematodes and bacteria is important for both organisms' survival (Akhurst & Boemare 1990; Forst & Clarke 2002; Hazir et al. 2003; Stock & Goodrich-Blair 2008). The nematodes require the bacterium for: 1) rapidly killing the host insect, 2) creating a suitable environment for nematode development via the production of antibiotics that supress competing microorganisms within the host, 3) transforming the host tissues into a food source and 4) serving as a food resource (Akhurst & Boemare 1990; Forst & Clarke 2002; Hazir et al. 2003; Stock & Goodrich-Blair 2008). The bacteria rely on the nematode for: 1) locating new insect hosts, 2) protection from external environmental factors, 3) penetrating into the insect host's haemocoel and 4) inhibiting the host's antibacterial proteins (Akhurst & Boemare 1990; Forst & Clarke 2002; Hazir et al. 2003; Stock & Goodrich-Blair 2008).

All stages of EPN are found within the insect host, except for the dauer juvenile (infective juvenile (IJ)) stage (Grewal et al. 2005; Lacey 2017). Life stages living within the host will undergo development and reproduction, with several generations possible, depending on the availability of nutrients. Once nutrients are depleted, new IJs will be produced and will exit the host cadaver (Figure 1.7) (Stock & Goodrich-Blair 2008). The IJs are the infective free-living stage that wait or actively search for the host (Grewal et al. 2005; Lacey 2017). The IJs of each family of EPN store the bacteria in different regions of the nematode. For *Steinernema,* the bacteria are stored in the anterior region of the intestine within a specialised bacterial receptacle; for *Heterorhabditis*, the bacteria are stored in the gut mucosa (Bird & Akhurst 1983; Ciche & Ensign 2003; Martens & Goodrich-Blair 2005; Ciche et al. 2008).

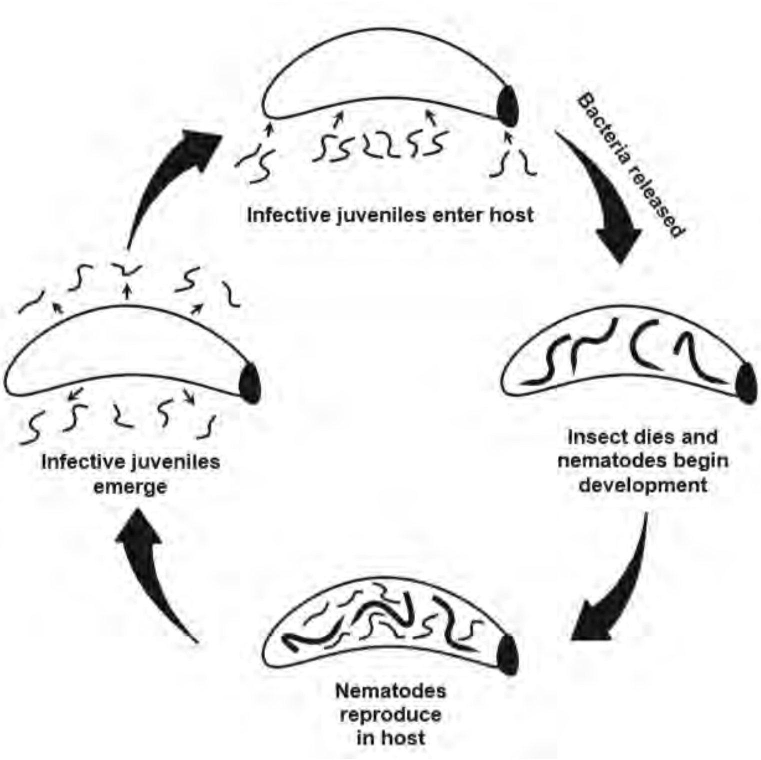


Figure 1.7. Movement of EPN between the host and free-living life stages (Taken from Shapiro-Ilan et al. 2017: Illustrated by Bill Joyner, USDA)

1.4.3 Generalised life cycle

The IJs are responsible for locating and invading the insect hosts. Different EPN species make use of one of three host-seeking strategies. The first method of host-seeking is ambushers, which are characterised as having low motility and predominantly remain near the soil surface, standing on their tails while awaiting a passing host (e.g. *S. carpocapsae*) (Shapiro-Ilan et al. 2017). The second method is cruisers, which are highly mobile nematodes which actively seek out their hosts via moving throughout the soil profile (e.g. *S. glaseri* and *H. bacteriophora*) (Shapiro-Ilan et al. 2017). Lastly most nematode species are intermediate foragers, which consists of a continuum between the ambusher and cruiser strategies (e.g. *S. riobrave* and *S. feltiae*) (Campbell & Gaugler 1997; Lewis et al. 1992; Campbell & Lewis 2002; Lewis 2002).

Environmental cues also effect the host-seeking abilities of EPN, be it chemical or physical (Kruitbos et al. 2010). The use of olfactory sensing of chemical volatiles plays a direct role in host location, because EPN will respond to different odours emitted by the insect host or plants

(Hallem et al. 2011; Dillman et al. 2012). For example, (E)-b-caryophyllene is a below ground plant signal/volatile that acts as an attractant for EPN when emitted from maize roots damaged by corn root worm (Ali et al. 2012; Hiltpold & Turlings 2012; Willett et al. 2015). Physical cues, such as vibrations and electromagnetic signals, can also assist IJs with navigating through the soil profile to locate hosts (Torr et al. 2004; Ilan et al. 2013). Group behaviour, another form of physical cue, is the process whereby the IJs move and infect hosts as a group which also contributes to successful host location and infection (Fushing et al. 2008; Shapiro-Ilan et al. 2014a).

Once a host has been successfully located, the first line of host defences (cuticle and peritrophic membrane of the midgut and respective epithelia) needs to be overcome. The IJs enter the host via spiracles, the anus, oral cavity and penetration of intersegmental membranes (Figure 1.8) (Lacey 2017; Shapiro-Ilan et al. 2017). The breaching of the host barriers triggers the host cellular and humoral immune responses (Castillo et al. 2011). Once inside the host, the IJs penetrate the haemocoel and release their mutualistic bacteria (Figure 1.8) (Lacey 2017; Shapiro-Ilan et al. 2017). The release of the bacteria differs between *Steinernema* and *Heterorhabditis* i.e. through the anus and through the mouth, respectively (Ciche & Ensign 2003; Ciche et al. 2008). The IJs and/or the symbiotic bacteria are able to overcome the insect defences by releasing virulence factors such as toxins, proteases and phenoloxidase inhibitors (Burman 1982; Laumond et al. 1989; Forst et al. 1997; Simões et al. 2000; Balasubramanian et al. 2009; Waterfield et al. 2009; Jing et al. 2010). The bacteria then kill off the host as a result of toxaemia or septicaemia 48 h after infection (Gulcu et al. 2017). The host tissues are metabolised by the bacteria and the IJs moult and begin feeding on bacterial cells and the metabolised host tissues (Figure 1.8) (Gulcu et al. 2017; Lacey 2017; Shapiro-Ilan et al. 2017).

The IJs will then undergo development which compromises of six life stages: egg, juvenile: one (J1), two (J2), three (J3), four (J4) and the adult (Gulcu et al. 2017). Reproduction differs between *Steinernema* and *Heterorhabditis*, whereby *Steinernema* are amphimictic and a male and female IJ need to enter the host for reproduction to occur whilst, *Heterorhabditis* are hermaphroditic and can reproduce when only one IJ has entered a host (Gulcu et al. 2017). *Heterorhabditis* have also been shown to produce both hermaphroditic and amphimictic progeny in new generations within a host (Koltai et al. 1995). The number of generations that occur within a single host depends on the availability of nutrients, in most cases two to three generations occur (Figure 1.8) (Gulcu et al. 2017; Lacey 2017; Shapiro-Ilan et al. 2017).

When the nutrients have been depleted, J2 develop into J3, commonly referred to as IJs and collect bacteria cells in preparation to exit the host (Gulcu et al. 2017; Shapiro-Ilan et al. 2017). The number of IJs produced is dependent on the insect host's size and the nematode species (Shapiro-Ilan et al. 2017). For example, when two nematode species *Steinernema riobrave* (average body length of 622 µm) and *S. glaseri* (average body length of 1133 µm) individually infect *Galleria mellonella* (Lepidoptera: Pyralidae), 300 000 IJs per host and 50 000 IJs per host are produced respectively, based on the differences in the IJs' body length (Grewal et al. 1994; Stock & Hunt 2005). Once the IJs are produced they sequester the bacterial cells to establish the symbiosis and then exit the host through openings (mouth, anus and spiracles) and search or wait for a new insect host (Figure 1.8) (Shapiro-Ilan et al. 2017).

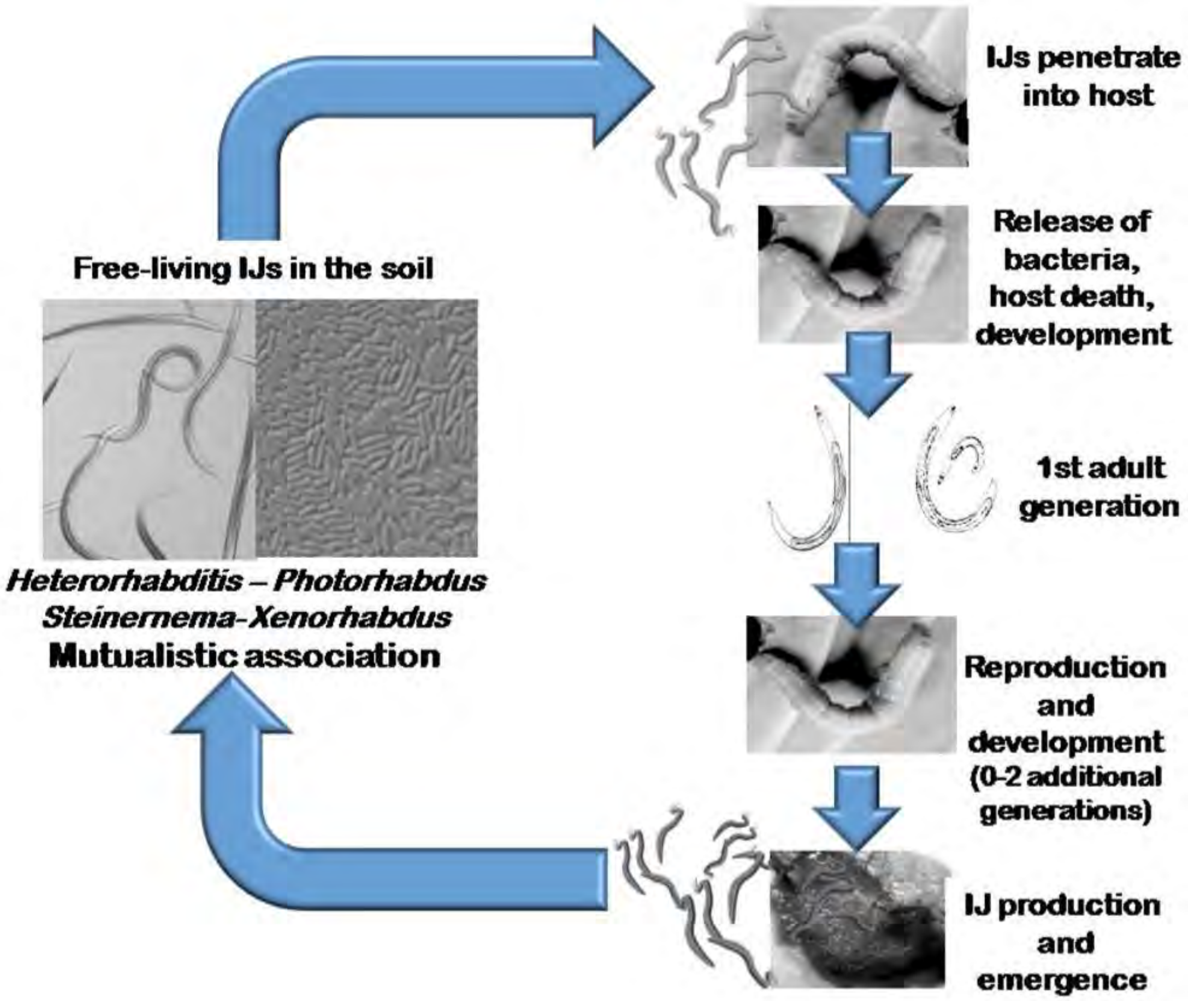


Figure 1.8. Generalised life cycle of entomopathogenic nematodes (Taken from Stock & Goodrich-Blair 2008).

1.4.4 Use as MCA

Entomopathogenic nematodes are used as microbial control agents (MCA) for augmentative biological control programmes and have been cultured commercially for 30 years, with at least 13 products commercially available worldwide (Georgis 2002; Poinar & Grewal 2012; Kaya et al. 2006; Lacey et al. 2015; Lacey 2017). Since the 1970s the number of commercially available EPN products has increased (Malan et al. 2011). EPN facilities such as "The Nematode Farm" at Berkeley was created in 1981 to control garden pests through the production of EPN products. The facility produced *S. glaseri, S. carpocapsae* and *H. bacteriophora,* from wax moth (*Galleria mellonella*) (Poinar & Grewal 2012). EPN were also being produced by Biotechnology Australia in 1983, which created Otinem, which was used to target black vine weevils (Poinar & Grewal 2012). In the 1990s research into EPN commercialisation increased, and protocols were developed to standardise experiments so that reliable results were obtained (Georgis et al. 2006). The movement away from chemical pesticides also facilitated the increase in EPN research (Georgis et al. 2006).

Although commercialisation processes for EPN had been investigated and developed, sales and research remained limited for EPN as biological control agents (Georgis et al. 2006). The delayed use of EPN products can be attributed to factors such as cost, formulation, shelf life, efficacy (in some cases not as good as available insecticides), application requirements and limited knowledge about EPN use in IPM programmes with regard to their efficacy in field trials and cost benefits (Georgis et al. 2006). However, EPN still remain important biological control agents for many insect pests in the agricultural industry because they are able to reach cryptic habitats, are safe for humans and vertebrates, have a high reproductive ability, persist within the environment and (once mass production protocols have been developed) are easy to produce (Malan et al. 2011; Shapiro-Ilan et al. 2017). EPN also have a diverse host range and certain species are known to infect insects across multiple orders (Poinar 1979; Klein 1990). Despite their high host range EPN are relatively safe for non-target organisms and have no effect on humans (Akhurst & Smith 2002; Ehlers 2005).

EPN from both the genera *Heterorhabditis* and *Steinernema*, are distributed throughout the globe (Poinar & Grewal 2012). Therefore, bioprospecting/baiting for new EPN species/isolates can be carried out in the locations where the target insect pest originates. However, most of the available EPN products originated from the northern hemisphere and only a few products are available in Africa. The idea of using commercially available but exotic EPN as control agents is widely debated. One of the arguments is against the use of exotics based on their potential to negatively impact the ecosystems they have been introduced into by outcompeting native EPN or impacting non-target insects (Malan et al. 2011; Millar & Babercheck 2001; Alpert 2006). Restrictions on the use of exotic EPN also poses another reason why exotics have been argued against (Koppenhöfer & Kaya 1997). The other side of the exotic's argument is for their use in non-native ecosystems. A review by Ehlers (2003) argues that research has shown that the displacement of native species by exotics is unlikely to occur and is dependent on their EPN/host relationship. If persistence of exotics does occur due to host availability and climate matching, then it is believed that the exotic and the native EPN will merely co-exist. The pro exotics argument has also show that non-target implications are minimal and only affect part of the population due to special restrictions (Ehlers 2003).

However, EPN are incredibly sensitive to environment factors as mentioned above, and the exotic EPN may not be able to withstand local environmental conditions with reductions in performance and virulence being observed (Alpert 2006; Zenni & Nunez 2013). In sub-Saharan Africa, harsher environmental conditions (compared to the Northern Hemisphere) may reduce the efficacy and/or persistence in the native ecosystem. South Africa, in particular, also has regulations in place to monitor and restrict the import of exotic species under the amendment of Act 18 of 1989 under the Agricultural Pest Act 36 of 1947 (Malan et al. 2011). Therefore, bioprospecting for native EPN species is important for controlling pest populations and maintaining the native ecosystems integrity. For the purpose of this thesis, native EPN species will be used.

1.4.5 EPN in South African citrus

Due to the problems surrounding exotic EPN and the strict regulations with introducing exotic species, bioprospecting for local South African EPN species is important for the future of IPM programmes. South African EPN species are understudied with minimal surveys conducted, and it remains the same for the rest of Africa (Malan et al. 2006; Hatting et al. 2019). EPN species that have been found for the first time in Africa are: *Steinernema yirgalemense* (Ethiopia) (Nguyen et al. 2004), *Steinernema karii* (Kenya) (Waturu 1998) *Steinernema taysaerae* (Egypt) (Shamseldean et al. 1996), *Heterorhabditis bacteriophora* and *Heterorhabditis indica* (new strains: Kenya) and reports of *Steinernema yirgalemense, Steinernema karii* and *Steinernema weiseri* (Central rift valley, Kenya) (Mwaniki et al. 2008; Malan et al. 2011). In South Africa, the following new EPN species have been discovered: *Steinernema khoisanae* (Mekete et al. 2005), *Steinernema citrae* (Stokwe et al. 2011) and *H. safricana* (Malan et al. 2006). This makes a total of eight *Steinernema* species recorded in South Africa: *S. citrae* (Stokwe et al. 2011), *S. khoisanae* (Nguyen et al. 2006), *S. fabii* (Abate et al. 2018), *S. innovationi* (Çimen et al. 2015), *S. yirgalemense* (Malan et al. 2011), *S. sacchari* (Nthenga et al. 2014), *S. tophus* (Çimen et al. 2015) and *S. jeffreyense* (Malan et al. 2016) (Malan & Moore 2016). A total of four *Heterorhabditis* species have been recorded in South

Africa: *H. bacteriophora* Poinar, 1976 (Malan et al. 2011), *H. safricana* (Malan et al. 2006), *H. zealandica* Poinar, 1990 (Malan et al. 2006) and *H. noenieputensis* (Malan et al. 2012) (Malan & Moore 2016). Despite these discoveries only one commercially available EPN product was available in South Africa, Cryptonem™ (River Bioscience, South Africa) which comprises of *Heterorhabditis bacteriophora* Poinar (Malan et al. 2006). However, its registration had been suspended due to the exotic strain used (produced in Germany (e-nema) (S.D. Moore, pers. comm.). More recently, both Cryptonem™ and a new *S. feltiae*-based product have been approved for registration but are not yet commercially available (Moore 2021).

Six of the native EPN species have been found to be virulent against FCM larvae/pupae (*S. khoisanae, S. citrae S. yirgalemense, S. jeffreyense*, *H. zealandica*, *H. bacteriophora* and *H. noenieputensis*) and adults (*S. yirgalemense* and *S. khoisanae*) (Malan et al. 2011, 2016; Steyn et al. 2019a, b). This thesis focuses on three of these EPN species; *S. yirgalemense, S. jeffreyense* and *H. noenieputensis*, because these species have been considered as the most promising candidates against FCM (Malan et al. 2011; Steyn et al. 2017; Malan & Moore 2016; Manrakhan et al. 2013; Steyn et al. 2019a, b).

1.5 INTERACTIONS

The soil community is species-rich and includes organisms such as viruses, bacteria, fungi, protozoans, collembola, tardigrades, and arthropods. The organisms within the soil community make up different feeding guilds and occur across different trophic levels (Scheu 2002). The soil foodweb is complex and offers a variety of ecosystem services such as nutrient and mineral reservoirs, manipulation of the soil structure, reducing pollutants and the regulation of pest populations (Doran & Parkin 1994; Kennedy & Smith 1995; van Straalen & van Gestel 1998; Ferrisa et al. 2001).

The regulation of pest populations is part of the top-down pressure within trophic levels, whereby prey populations are regulated by predators/parasitoids (Scheu 2002). For example, pest insects, such as FCM, are regulated by natural enemies (e.g. EPN and EPF) within the environment. When investigating the relationship between predator and prey, it is also important to investigate the relationship between different predators to accurately assess how the biodiversity of an ecosystem interacts. Henceforth, the term ‘predator’ refers to predators including pathogens and parasitoids.

The relationship between these predators can be antagonistic, whereby the effects of both organisms combined is less than the effects of the individual organisms acting alone. Antagonistic factors that affect soil communities include competition, natural enemies, and antibiosis (Kaya & Koppenhöfer 2010). Antagonistic interactions can be direct (predation by natural enemies) or indirect (competition for resources and antibiosis). Nematophagous fungi that prey upon nematodes within the soil, is an example of direct antagonism (Jansson & Lopez-Llorca 2004; Kaya & Koppenhöfer 2010). Indirect antagonism through competition for resources (hosts) and antibiosis, occurs within the soil community between predators and can be inter- and intra-specific (Kaya & Koppenhöfer 2010). An example of an intra-specific antagonistic interaction is two *Steinernema* species which have cohabited a host and, over time, one species will outcompete the other (Kaya & Koppenhöfer 2010). An inter-specific antagonistic interaction would be competition between EPN from different genera that have cohabited the same host. For example, *Steinernema* species have been found to exclude *Heterorhabditis* species from entering the same host (Kaya & Koppenhöfer 2010). This is caused by the production of bacteriocin(s) by *Steinernema*-associated *Xenorhabdus* bacteria. These bacteriocins preventing the growth of *Heterorhabditis*-associated *Photorhabdus* bacteria within the same host (Kaya & Koppenhöfer 2010). Indirect antagonistic relationship between soil predators can occur between EPN, viruses, bacteria and fungi (Kaya & Koppenhöfer 2010).

However, not all interactions between soil predators are antagonistic. Synergism between predators can occur and can be manipulated to improve IPM programmes for pest population control. The term ‘synergism’ is used when the combined effect of multiple organisms is greater than the sum of the single organism (Piggott et al. 2015). These interactions require a ‘stressor’, which causes the sum of the combined interactions effects to be greater than the individual effects. A stressor in this context could be products released by the primary infector, which weakens or compromises the host’s immune system, allowing for a rapid secondary infection to occur because the host is now more susceptible, (Piggott et al. 2015; Wakil et al. 2017). In order for a synergistic relationship to occur, the stressor produced by the primary infector, needs to be beneficial to the secondary infector. This will allow for the combined effects of both infectors to be greater than the effects of the individual infections (Piggott et al. 2015). Hence, synergistic interactions can increase the control efficacy of IPM programmes. Other benefits of using combinations of control agents are that a variety of modes of action can be used to combat the insect host and its defence/immune system (Devi 2019). In addition, multiple life stages could be targeted at a time when combined control agents are released (Devi 2019).

IPM programmes involve the combination of multiple control strategies to target pest populations. Understanding the interactions between multiple control products is key to improving and efficiently implementing IPM strategies. If interactions between two control agents can be more effective (synergistic) than the products alone, an increase in pest population control can be achieved. The interactions between combinations of different EPN and EPF have been investigated and potential synergistic relationships have been found in both greenhouse and field trials. In all cases, the EPF was found to be the stressor that facilitates the increased control efficacy when EPN and EPF are combined (Ansari et al. 2004). In Ansari et al. (2004) the EPF *M. anisopliae* was combined (separately) with two EPN species, *H. megidis* and *S. glaseri,* against *Hoplia philanthus* Füessly (Coleoptera: Scarabaeidae). They found that synergistic relationships between the EPF and the EPN were achieved, if the EPF was applied first and then the EPN were applied after a period of time (Ansari et al. 2004). The process of applying the stressor first, is an important step in achieving synergism between two control agents. Timing of application also plays a role when targeting specific life stages. For example, synergistic effects are only achieved between *M. anisopliae* and the bacterium *Serratia entomophila* with early instars, not in later instar grass grub larvae of *Costelytra zealandica* (White) (Ansari et al. 2004). Other examples of EPN and EPF interactions include the synergistic relationship found between *H. bacteriophora* Poinar combined with *Beauveria bassiana* (Vuill) Balsamo (Barbercheck & Kaya 1991) and *S. carpocapsae* (Weiser) combined with *Beauveria brongniartii* (Saccardo) Petch used to target white grubs *Ectinohoplia rufipes* and *Exomala orientalis* (Coleoptera: Scarabaeidae) (Choo et al. 2002).

In the case of EPF as stressors, hosts infected with fungal infections show reduced signs of feeding, which negatively impacts the host's internal environment (Ansari et al. 2004). This leaves the host susceptible to secondary infections because the physiology, morphology and behaviour linked to host defences have been compromised by the stressor (Gaugler et al. 1994; Wang et al. 1995; Ansari et al. 2004). The amount of EPF (stressor) is another factor that influences the interaction between EPN and EPF. Ansari et al. (2004) also found that high concentrations of *M. anisopliae* conidia inhibited EPN reproduction. This is supported by Mehdi et al. (2018), who examined the relationship between *B. bassiana* and *H. bacteriophora* on Red Palm Weevils (RPW) *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) and found that low conidial concentrations increase the efficacy of combined EPN and EPF efforts. Higher concentrations of the fungus within the host gut were speculated to prevent the symbiotic bacteria's ability to multiply, directly affecting the reproduction success of the invading nematode (Ansari et al. 2004). EPF can also produce toxins, which may also inhibit the EPN symbiotic bacteria (McCoy et al. 1988; Vey et al. 2001). However, EPN can also inhibit fungal infection through the production of antibiotics by their symbiotic bacteria, seen in *Xenorhabdus* species, such as *Photorhabdus luminescens* (Kaya & Gaugler 1993; Ansari et al. 2004). This again emphasises the importance of timing when applying EPF and EPN together, if the EPF does not have sufficient time (one to seven days) prior to EPN infection, EPN can exclude the EPF (Kaya & Gaugler 1993).

1.6 RESEARCH AIMS

Previous research has highlighted four MCA as potential controls for FCM in the South African citrus industry. Based on these findings and the present literature which suggests that synergistic interactions between MCA can result in increased control, the types of interaction of three EPN species and one EPF species was assessed. Understanding the interactions between control agents is a fundamental part of IPM programmes, with no exception for FCM. This is a novel study which investigates the types of interactions that take place between three EPN species *S. yirgalemense* 157-C*, S. jeffreyense* J194 and *H. noenieputensis* 158-C when combined with the EPF isolate *M. anisopliae* FCM Ar 23 B3. This information will help determine when the EPN and EPF species (once developed into commercial products) should be applied and whether combined or sequential application is necessary to achieve a successful reduction in FCM soil-dwelling populations within orchards.

Chapter 2

CONTROL POTENTIAL OF SELECT EPN AND EPF ISOLATES AGAINST FCM FIFTH INSTARS

2.1 INTRODUCTION

Thaumatotibia leucotreta, false codling moth (FCM), is one of the key citrus pests in South Africa and is listed as a phytosanitary pest for important international export markets (Adom et al. 2020, Moore 2021). Control strategies for this pest species comprise chemical insecticides, manual and mechanical control and biological control agents, which are combined to form part of an IPM programme for FCM in South Africa. To date, no control options are commercially available for the management of the soil-dwelling life stages, fifth instar and pupae, of this important citrus pest (Moore 2021). Recently two EPN products (*Heterorhabditis bacteriophora* and *Steinernema feltiae*) were approved for combating soil-dwelling FCM life stages, but their commercial availability is pending (Moore 2021).

Control of these soil-dwelling insect pests may be achieved using soil-dwelling pathogens, namely EPN and EPF. As previously outlined in Chapter 1, EPN and EPF have been shown to be successful control agents of insect pests, of both soil- and non-soil dwelling species. Previous research into EPF and EPN as control agents for soil-dwelling insect pests primarily focuses on Coleoptera and Lepidoptera species (Georgis & Gaugler 1991; Klein 1993; Thurston et al. 1994; Ansari et al. 2003; Ansari et al. 2004; Barbercheck & Kaya 1990; Choo et al. 2002; Nueno-Pallero et al. 2018). This research has gone further, by investigating the types of interactions that might occur between EPF and EPN when controlling an insect pest, to improve pest control programmes (Nueno-Pallero et al. 2018). This interaction will be addressed in more detail in Chapter 3.

In this study, EPF and EPN which had shown promising results in previous laboratory experiments, were investigated for the control of the first soil-dwelling FCM life stage, fifth instars. With respect to EPF, two fungal isolates *B. bassiana* G Ar 17 B3 and *M. anisopliae* FCM Ar 23 B3, were selected as possible control agents for FCM by Goble et al. (2011) and Coombes (2013). However, Acheampong (2019) found that *B. bassiana* G Ar 17 B3 showed a reduced virulence relative to the field trials conducted by Coombes et al. (2016). Therefore, only the fungal isolate *Metarhizium anisopliae* FCM Ar 23 B3 was selected for further testing and is the EPF isolate that was investigated for the remainder of this thesis.

Malan et al. (2011, 2016) and Steyn et al. (2019a), showed that the EPN species *Steinernema yirgalemense, Steinernema jeffreyense* and *Heterorhabditis noenieputensis* were recorded to infect and successfully cause FCM mortality. *Steinernema yirgalemense* (Rhabditida: Steinernematidae) was first identified in Ethiopia and described by Nguyen et al. (2004). *Steinernema yirgalemense* belongs to the 'biocornutum-group' of EPN, which are characterised by the two distinct horn-like structures on the cephalic region (Nguyen et al. 2004). The infective juveniles (Figure 2.1) are 635 µm in length and the species description can be found in Nguyen et al. (2004). *Steinernema yirgalemense* is associated with the bacterial symbiont *Xenorhabdus indica* (Ferreira et al. 2016a). In South Africa, *S. yirgalemense* was first recorded by Malan et al. (2011), recovered from soil samples collected from a citrus orchard in Nelspruit, Mpumalanga.

In South African studies conducted on insect pests of citrus, grapes and deciduous fruit, *S. yirgalemense* has been found to be highly virulent compared to other EPN species (Malan et al. 2011; Le Vieux & Malan 2013) and has shown to be the most promising EPN for controlling FCM (Malan et al. 2011) and codling moth (De Waal et al. 2011). Bioassays conducted by Malan et al. (2011) using *S. yirgalemense* to control FCM, showed that *S. yirgalemense* is considered a top candidate for the control of FCM because it can infect the larvae, pupae and emerging adults. *Steinernema yirgalemense* induced 100% and 74% mortality of larvae and pupae respectively, at concentrations of 50 IJs/larvae and 200 IJs/pupae (Malan et al. 2011).

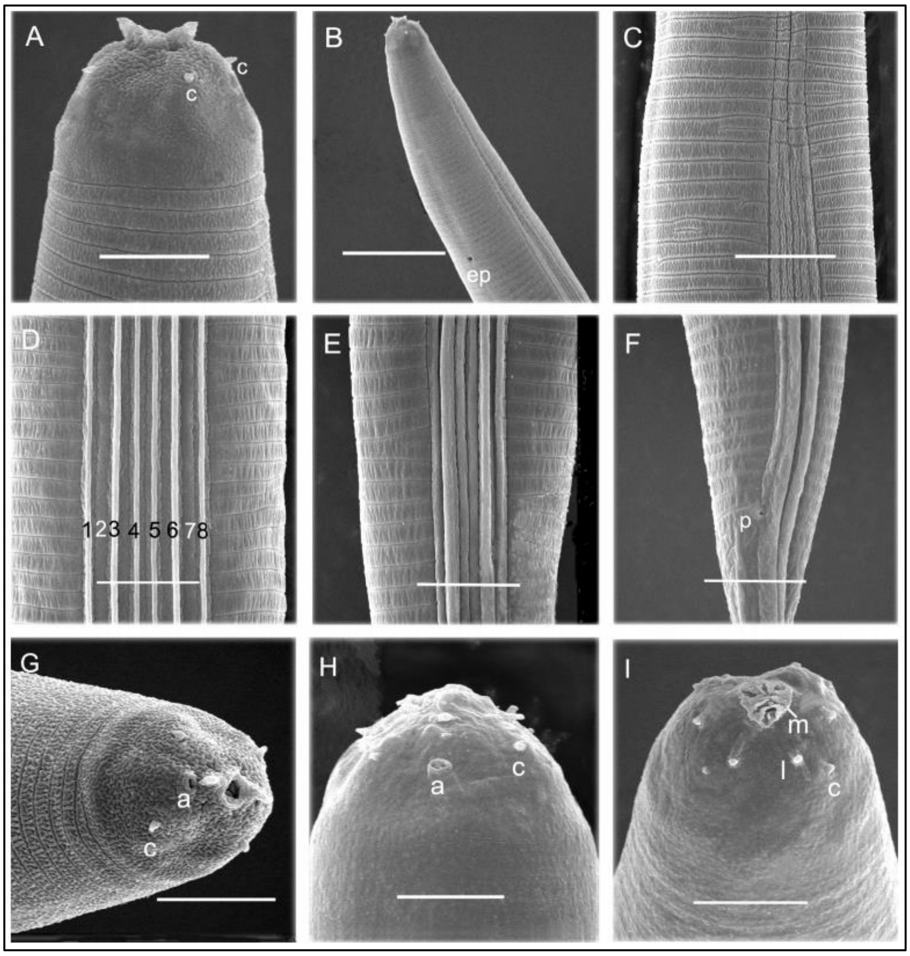


Figure 2.1. *Steinernema yirgalemense* n. sp. infective juvenile under SEM. Scale bars: (A) 3.75 µm, (B) 15 µm, (C & D) 6.67 µm, (E & F) 6.00 µm, (G) 4.28 µm and (H) 2.73 µm (Taken from Nguyen et al. 2004).

Steinernema jeffreyense (Rhabditida: Steinernematidae) was first isolated in Jeffrey's Bay, Eastern Cape Province, South Africa (Malan et al. 2016). The isolate is recorded as *Steinernema jeffreyense* J194 (Stokwe 2016). The morphological and phylogenetic characterisation of *S. jeffreyense* is described by Malan et al. (2016). The infective juveniles have tapered, slender bodies with an average body length of 926 µm (Figure 2.2) (Malan et al. 2016). *Steinernema jeffreyense* has a symbiotic association with the bacteria *Xenorhabdus khoisana* and has been successfully mass produced *in vitro* at Stellenbosch University, South Africa (Dunn et al. 2019). In field trials conducted by Steyn et al. (2019a), *S. jeffreyense* induced significant weekly reductions of FCM larvae in the field after application to the soil surface.

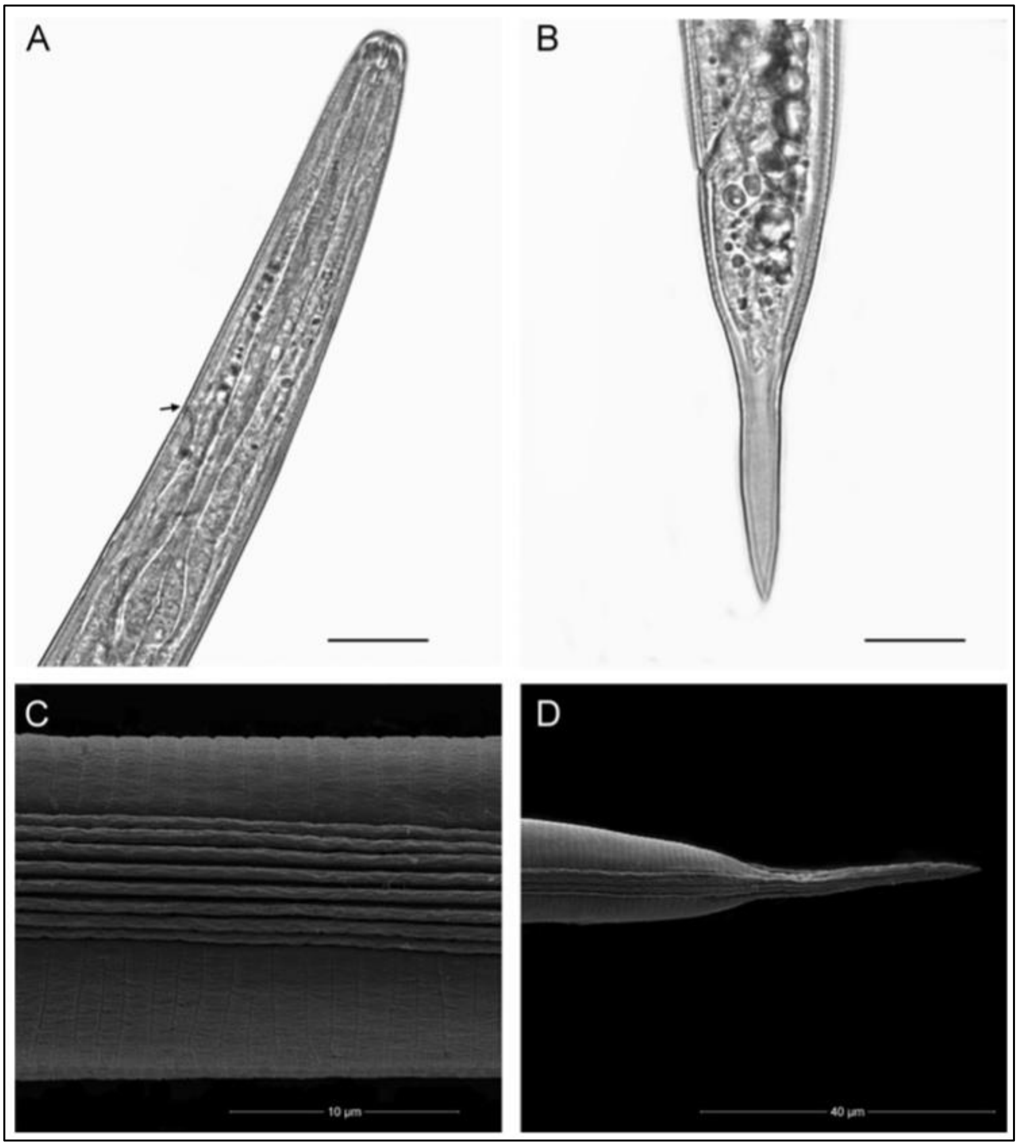


Figure 2.2. *Steinernema jeffreyense* n. sp. infective juvenile. Scale bar: (A & B) 20 µm, (C) 10 µm and (D) 20 µm (Taken from Malan et al. 2016).

Heterorhabditis noenieputensis (Rhabditida: Heterorhabditidae) is a relatively new species, first recorded in Noenieput, Northern Cape Province, South Africa (Malan et al. 2012; Stokwe 2016). The isolate was recorded as *H. noenieputensis* SF669. Another isolate was also identified during field trials in the Nelspruit area (Mpumalanga Province, South Africa), by Malan et al. (2011) and is recorded as isolate *H. noenieputensis* 158-C. This isolate was selected for testing in this thesis. The bacteria associated with *H. noenieputensis* is *Photorhabdus luminescens* subsp. *noenieputensis* (Ferreira et al. 2013). The morphological and phylogenetic characterisation of *H. noenieputensis* is described by Malan et al. (2012). The IJs are long and slender with a body length of 536 µm (Figure 2.3) (Malan et al. 2012). *Heterorhabditis noenieputensis* produce first generation hermaphrodite females, whilst later generations are amphimictic females and males. These are described and illustrated in Malan et al. (2012). The current method for harvesting *H. noenieputensis* is by recycling the EPN through *Galleria mellonella* (L.) (greater wax moth) at Stellenbosch University (Malan et al. 2011; A.P. Malan, pers. comm.). In screening studies by Malan et al. (2011, 2012), *H. noenieputensis* 158-C was found to successfully infect FCM late instar larvae (< 70% mortality) and pupae (27% mortality) at concentrations of 50 IJs and 200 IJs per larva, respectively.

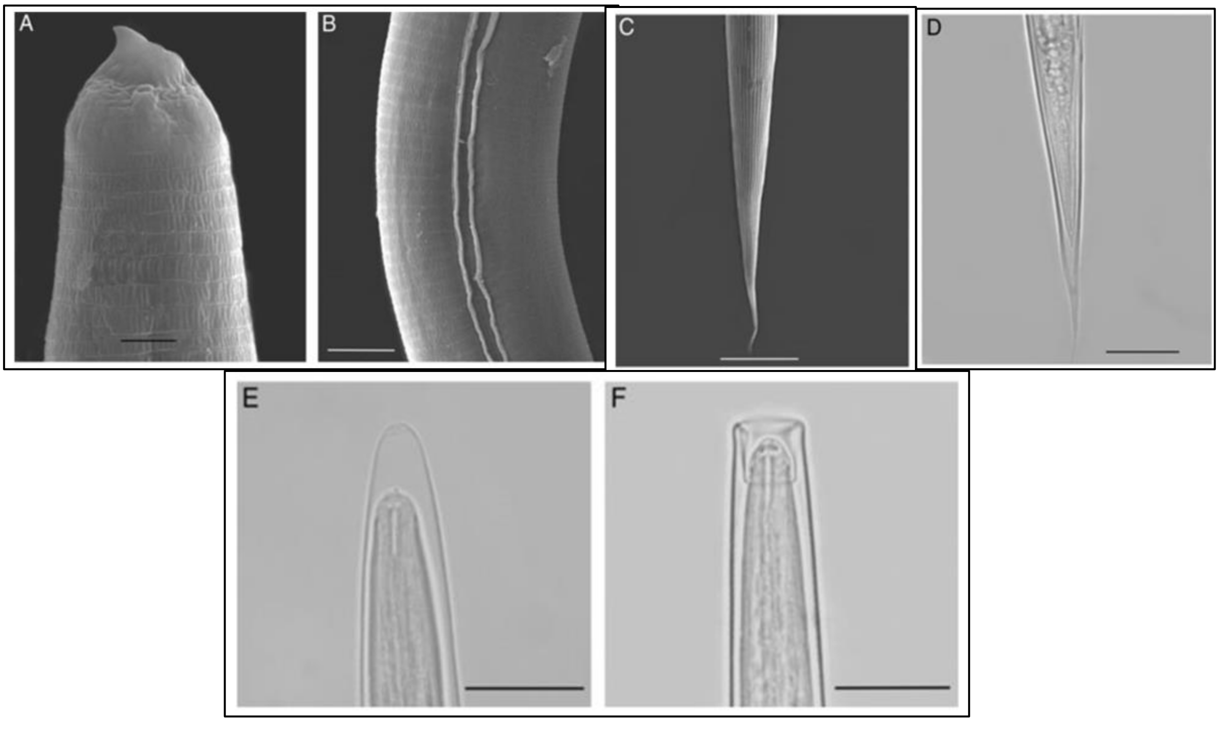


Figure 2.3. *Heterorhabditis noenieputensis* n. sp. infective juvenile (IJ). Scale bars: (A) 2 µm, (B) 5 µm, (C) 20 µm and (D – F): 20 µm (Taken from Malan et al. 2012).

Based on these findings, which used a single EPN dose to yield close to 100% mortality, the three EPN species were selected for further screening to determine which amongst them is the most virulent by conducting dose-response bioassays. Therefore, the aim of this chapter was to determine which EPN species was more virulent against FCM fifth instar larvae by conducting single MCA application with a uniform bioassay protocol at the recommended application rates to confirm infectivity and virulence. Secondly, this chapter aimed to establish the lethal concentration (LC) values required to kill 50, 70 and 90% of the FCM fifth instars for all three EPN species and the EPF isolate. These values are currently unknown for the EPN species, and although known for the EPF isolate used in this study, the bioassay methodology to be used in the preceding chapter differs to the soil bioassays previously conducted to establish LC_{50} and LC_{90} values (Coombes et al. 2015). These results will aid other laboratory research aimed at further investigating the efficacy of microbes for possible biological control agents of soil-dwelling life stages of FCM.

2.2 MATERIALS AND METHODS

2.2.1 Insect cultures

Fifth instar FCM were obtained from a laboratory-reared colony established at Rhodes University. Larvae were reared on artificial diet according to Moore et al. (2014), using 350 ml glass jars plugged with cotton wool, in which pupation occurs (Figure 2.4). The upward movement of larvae into the cotton wool is indicative of fifth instars.

Figure 2.4. False codling moth rearing jars with artificial diet (Photo credit: S. Prinsloo).

2.2.2 Microbial cultures

2.2.2.1 Entomopathogenic nematodes

Steinernema yirgalemense 157-C, GenBank accession number EU625295 (Malan et al. 2011) and *S. jeffreyense* J194, GenBank accession number KC897093 (Stokwe 2016), IJs were obtained from *in vitro* cultures from Stellenbosch University. *Heterorhabditis noenieputensis* 158-C, GenBank accession number JN620538 (Stokwe 2016), IJs were also obtained from Stellenbosch University, but were cultured *in vivo*, using wax moth larvae (*Galleria mellonella* L. (Lepidoptera: Pyralidae). Fresh IJ cultures of all three EPN species were maintained by *in vivo* culturing using fifth instar FCM larvae obtained from Rhodes University. The EPN were passed through these insects every two weeks to ensure viability or, if cultures were older than two weeks, fresh nematodes were ordered from Stellenbosch University. Infected larvae were kept at room temperature in modified White traps for collection of fresh IJs. The harvested IJs were then placed in sterile vented culture flasks and stored at room temperature. The flasks were agitated weekly to aerate the IJs (A.P. Malan, pers. comm.).

2.2.2.2 Entomopathogenic fungus *M. anisopliae* FCM Ar 23 B3

The PPRI Fungal Accession number for *M. anisopliae* FCM Ar 23 B3 is PPRI 9561, the Rhodes code is FCM Ar 23 B3 (Goble et al. 2011) and the GenBank accession number is KF83418 (Chartier Fitzgerald et al. 2016). The isolate was originally obtained from Arundel (33º30′57″ S; 25º39′11″ E), from loamy soil within a cultivated citrus orchard in April 2008 (Goble et al. 2011). *Metarhizium anisopliae* FCM Ar 23 B3 was obtained from laboratory cultures stored at Rhodes University (Figure 2.5). The fungal isolate was cultured on Sabouraud Dextrose Agar (SDA) supplemented with 50 mg/L chloramphenicol in a controlled environment (CE) room at 26 ± 1 °C on a 12 h photoperiod. For all experiments, 14 day old plate cultures were used (Figure 2.6). The isolate was passaged through fifth instar FCM at the beginning of a new experiment/replicate to maintain fungal viability and virulence. Plates that had been sub-cultured three times were discarded.

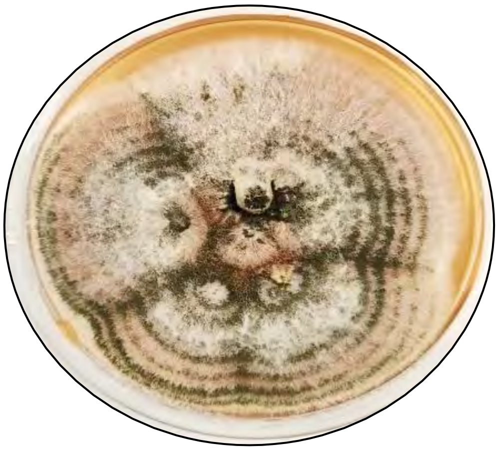


Figure 2.5. *Metarhizium anisopliae* FCM Ar 23 B3 growth plate on SDA media after 14 days (Photo credit: S. Prinsloo).

2.2.3 Preparation of microbial suspensions

2.2.3.1 Entomopathogenic nematodes

For each nematode species, 50 µl of the stock suspension was poured into a 50 ml plastic measuring cylinder, sealed and inverted five times to ensure the nematodes were mixed evenly in suspension. Three rows of five 10 µl droplets were then pipetted onto the lid of a petri dish to determine the number of nematodes in suspension. In each droplet, only actively moving nematodes were counted. The total number of nematodes for each row was recorded and then the average number of nematodes between the three rows was calculated. The desired concentration was then calculated using the formula (A.P. Malan, pers. comm.):

$$\left(\frac{x}{y} - 1\right) \times V = z$$

Where:

x = average number of IJs counted

y = desired number of IJs

z = necessary adjustments to original stock (removal or addition of sterile dH_2O)

V = stock volume

The EPN stock was then either diluted down with dH_2O if z = +ve, or the stock was reduced if z = -ve. The latter was achieved by allowing the IJs to settle at the bottom of the cylinder and siphoning off from the upper regions of the stock, which did not contain IJs. Each EPN suspension was used on the same day of preparation.

2.2.3.2 Entomopathogenic fungus *M. anisopliae* FCM Ar 23 B3

Fungal suspensions were prepared under a laminar flow hood, by flooding the plate with 20 ml of sterile distilled water, supplemented with 0.01% Tween® 20 and gently scraping the surface to release the conidia. The suspension was then collected from the plate into a 20 ml universal glass bottle and shaken vigorously to homogenise the suspension. Using a Helber bacteria counting chamber with Thoma ruling as viewed under a light microscope (400X), the concentration of the stock suspension was determined and then diluted to obtain a final concentration of 1×10^7 conidia/ml.

The following formula was used to determine the fungal stock concentration:

$$\frac{Dilution \times Spore\ count}{5 \times 10^{-8} \times 80}$$

A three-fold dilution series was used to prepare all remaining concentrations necessary for this study. Fungal viability was assessed by spread plating 100 µl of a 1×10^5 conidia/ml fungal suspension onto three SDA agar plates, supplemented with 50 mg/L chloramphenicol. The plates were placed in a box in complete darkness in a CE room at 26°C. The number of germinating spores was then determined by counting the first one hundred spores visualised and noting whether they had germinated (the presence of a germ tube) or not (Alves et al. 1998). The average percentage of germinating spores was calculated. The fungal suspension was considered viable if > 90% of the spores had germinated.

2.2.4 Efficacy of EPN mortality alone on FCM fifth instars

The three EPN species have been shown to be virulent against FCM. However, a single study that incorporates all three species using fifth instar FCM larvae has yet to be completed. Therefore, this study aimed at identifying the virulence of the three EPN species through bioassays in 24-well plates using a uniform concentration rate of 50 IJs/larvae for initial assessment and 50 IJs, 25 IJs, 12.5 IJs, 6.25 IJs and 3.125 IJs, for the subsequent dose-response bioassays. The dose response bioassays were necessary to determine further variations between the three EPN species virulence against fifth instar FCM, with the LC_{50}, LC_{70}, and LC_{90} values needed for use in the EPN and EPF interaction experiments (Chapter 3). Both the individual EPN species and the dose response bioassays follow the same experimental design, barring the differences in IJ concentrations (specifically for the dose response bioassays).

Sterile filter paper discs (12.7 mm) were placed in every alternate well to which 50 µl of the recommended dose was pipetted onto each disc. Fifth instar FCM were then added to each treated well. A glass lid was placed over the wells to prevent larval movement between wells. Five plates were used per EPN treatment (dose), with a total sample size of 60 larvae per treatment (dose). Each treatment was then grouped together with their respective five plates, using elastic bands, which were then placed into 2 L plastic containers lined with moist tissue paper and placed in a CE room at 26 ± 1 °C on a 12 h photoperiod for 48 h. A control treatment was included for both the individual and dose response bioassays whereby 50 µl of distilled water was applied to the wells. Thereafter, the same procedure as described above was followed.

After 48 h the nematode treatments (including the control) were individually assessed and the dead or living larvae were separated and surface sterilised by washing the larvae in a tea sieve with distilled water over a glass beaker. The dead larvae were placed onto moist filter paper inside petri dishes for a further 48 h; the same was done for the living larvae. The additional 48 h (96 h after inoculation) was included for two reasons. Firstly, surviving larvae may be infected with EPN towards the end of the initial 48 h period, and an additional 48 h is required to allow for death as a result of EPN infection (A.P. Malan, pers. comm.). Secondly, the additional 48 h gives the EPN time to replicate within the host and allowing the larva to first become heavily infected will ensure that the cause of death is accurately recorded to be as a result of EPN infection (A.P. Malan, pers. comm.).

Once the 96 h had passed, the dead larvae were first examined visually for a bacteria-induced colour change (Figure 2.6) (Griffin 2012). EPN infection was then confirmed by assessing the presence or absence of EPN within the larval cavity. An individual larva was placed in a petri dish under a dissection microscope (4 – 8X magnification) and, using fine forceps, the larva was pulled apart and the gut contents squeezed out. Distilled water was added to submerge the gut contents in liquid, which was then examined for the presence or absence of nematodes and recorded. The percentage mortality for each EPN species was then calculated.

Any larvae that had not succumbed to EPN infection 96 h post-treatment, were placed into glass vials that were plugged with cotton wool and left to pupate. Ten days after the first moth eclosed the experiment ended. The experiment was replicated three times for the individual dose and dose response bioassays.

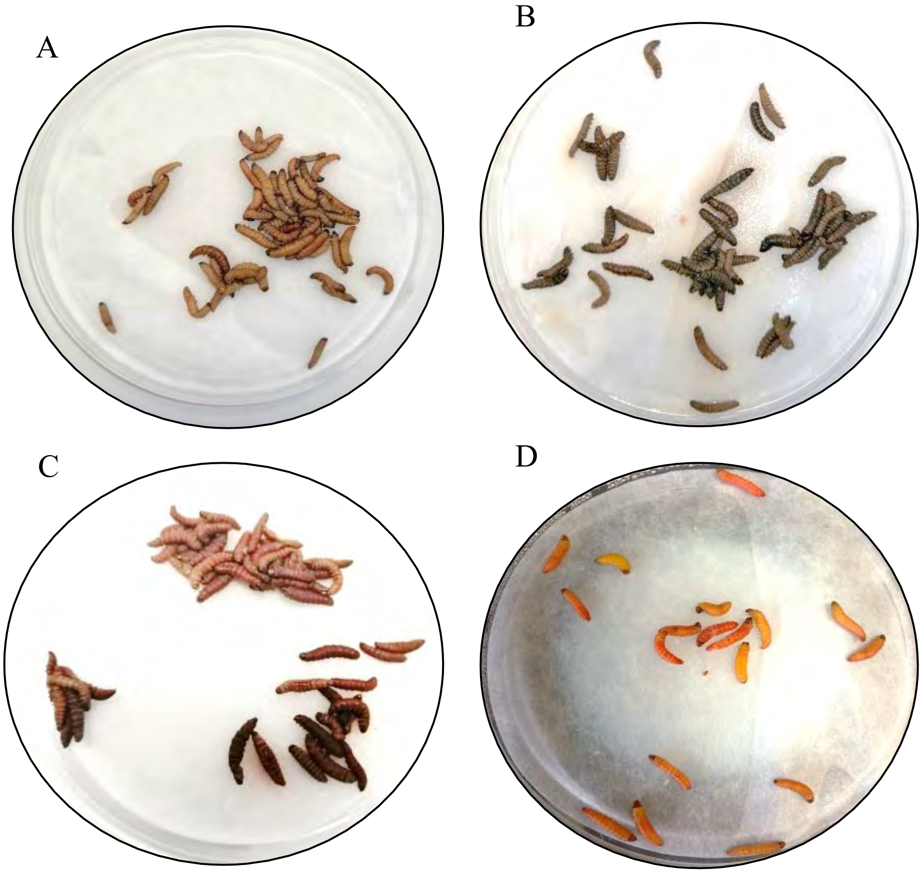


Figure 2.6. Fifth instar FCM larvae infected with EPN A-C: (A) *Steinernema yirgalemense* exhibiting a yellow colour change, (B) *Steinernema jeffreyense* exhibiting a black/brown colour change and (C) *Heterorhabditis noenieputensis* exhibiting a red/brown colour change, (D) an example of healthy fifth instar FCM exhibiting their natural pink colouration (Photo credit: S. Prinsloo, C. Coombes).

2.2.5 Efficacy of EPF alone on FCM fifth instars

Although the LC_{50} of this isolate has been established (Coombes et al. 2015), the bioassay procedure is different to that needed for conducting combined EPN and EPF experiments (Chapter 3). Furthermore, individual bioassays using a recommended application rate (1×10^7 conidia/ml) and dose response bioassays needed to be conducted for comparative purposes between the individual agents and to obtain the LC_{50}, LC_{70} and LC_{90} values for the combined bioassays (Chapter 3). For the dose response bioassays, a three-fold serial dilution was used which included seven doses: 1.4×10^4, 4.1×10^4, 1.23×10^5, 3.7×10^5, 1.11×10^6, 3.33×10^6 and 1×10^7 conidia/ml (Coombes et al. 2015) The experimental protocol was the same for the individual dose EPF isolate and dose-response bioassays.

Using 24-well bioassay plates, sterile filter paper discs (12.7 mm diameter) were placed in every alternate well. Each fifth instar FCM larva was dipped into the fungal suspension and a single larva was placed into a well. A control was included, dipping the larvae into 0.01% Tween® 20 solution. The wells were covered with a piece of glass and the plates were sealed with their corresponding plastic lids. Each treatment contained five bioassay plates, with a total of 60 larvae per treatment. The bioassay plates were grouped per treatment using an elastic band and placed into a 2 L plastic container, which was lined with moist paper towel. The plastic containers were placed in a CE room at 26 ± 1 °C and in the dark for 7 days.

On day 7, the larvae were examined and were separated into dead or alive. The dead larvae were surface sterilised using 70% ethanol (Coombes et al. 2013), placed onto moist filter paper in petri dishes and left to sporulate in a CE room to confirm fungal death (Figure 2.7). The surviving larvae were placed individually into glass vials that were plugged with cotton wool. The larvae were left to pupate, and the experiment was terminated 10 days after first eclosion was noted. The experiment was replicated three times for both the individual and dose response bioassays.

Figure 2.7. Fifth instar FCM infected with *Metarhizium anisopliae* FCM Ar 23 B3, showing sporulation (Photo credit S. Prinsloo).

2.2.6 Statistical analysis

A statistical analysis of the individual agents' percentage mortality was performed using R Studio, R version 3.6.2 (R Core Team 2019). The Abbott's formula for correcting mortalities was used to account for natural mortality (Abbott 1925):

$$Corrected\,mortality = \frac{\text{Treatment} - \text{Control}}{100 - \text{Control}} \times 100$$

A Shapiro-Wilks tests for normality was conducted and the individual control agent's percentage mortalities were subjected to an analysis of variance (ANOVA). If a significant difference was recorded a Tukey's post-hoc test: separation of means, was conducted ($P < 0.05$) (using the packages tidyverse (Wickham 2019), MuMIn (Barton 2020), dplyr (Wickham et al. 2020), FSA (Ogle et al. 2020), car (Fox & Weisberg 2019), agricolae (de Mendiburu 2020), multcomp (Hothorn et al. 2008) and janitor (Firke 2020)).

A Probit analysis was conducted on the dose response data using R Studio, R version 3.6.2 (R Core Team 2019) (using the "drc" function and the drc (Ritz et al. 2015) and Hmisc (Harrell et al. 2019) packages). The LC_{50}, LC_{70} and LC_{90} values for all three EPN species and the EPF isolate were determined from this analysis.

2.3 RESULTS

2.3.1 Screening of EPF and EPN control agents against FCM

The average percentage mortality of FCM fifth instars ranged between 91 and 96% for all EPN species with *S. yirgalemense* causing the highest percentage mortality (Figure 2.8). The EPF isolate, *Metarhizium anisopliae* FCM Ar 23 B3, induced an average of 80% mortality of FCM fifth instars. Statistical differences amongst treatments were found ($F_{(3,8)} = 6.433$; $P = 0.01587$), with the EPF isolate recording a significantly lower percentage mortality compared to two of the EPN species tested (Figure 2.8). Control mortality was always a result of handling error, never EPN nor EPF infection.

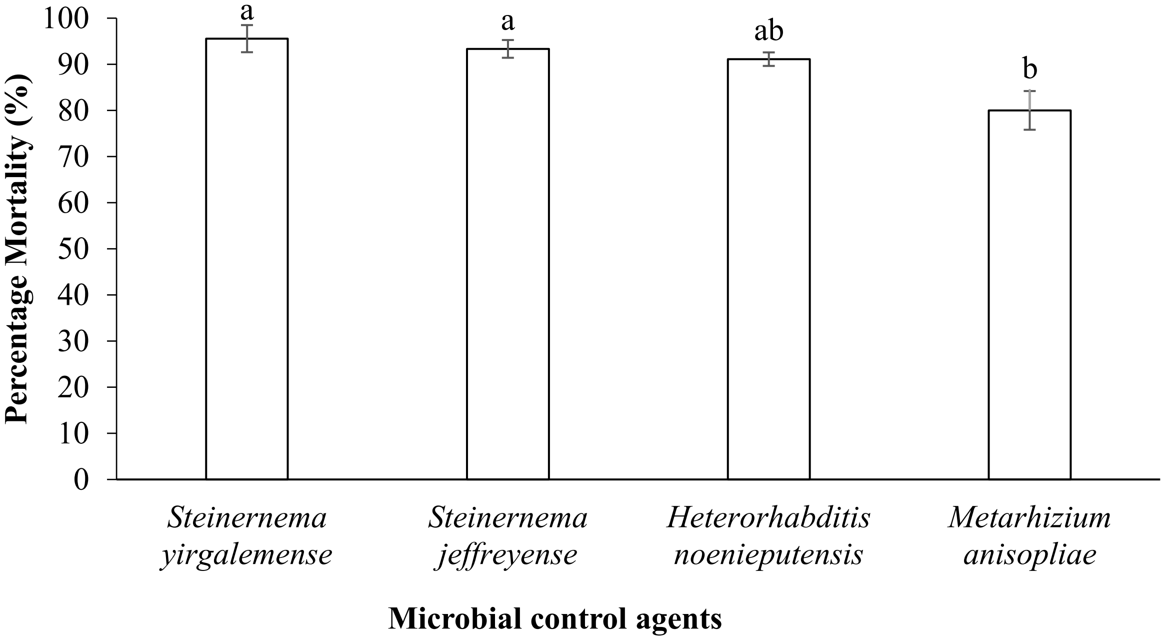


Figure 2.8. The mean percentage mortality (%) and standard error (± SE) of fifth instar FCM when applied with the individual microbial control agents: (EPN recommended concentration of 50 IJs/larva) *Steinernema yirgalemense, Steinernema jeffreyense* and *Heterorhabditis noenieputensis* and (EPF recommended concentration of 1×10^7 conidia/ml) *Metarhizium anisopliae* FCM Ar 23 B3. Different letters above the bars represent significant differences according to the Tukey's post-hoc test ($P < 0.05$) ($F_{(3,8)} = 6.433$; $P = 0.01587$). The Abbotts (1925) formula was used to correct for natural mortality; the EPN and EPF control survival was greater than 80%.

2.3.2 EPN and EPF Dose response bioassays

Dose response bioassays were conducted for the three EPN species and the *M. anisopliae* against fifth instar FCM in 24-well plates. Regression lines for each control agent were fitted (Figure. 2.9). *S. yirgalemense* 157-C: y = 0.5401 (SE of slope = 0.05) x + 0.0856, *S. jeffreyense* J194: y = 0.5847 (SE of slope = 0.05) x + 0.2223, *H. noenieputensis* 158-C: y = 0.7576 (SE of slope = 0.05) x + 0.0480 and *M. anisopliae* FCM Ar 23 B3: y = 3.9770 (SE of slope = 0.21) x + 2.4998.

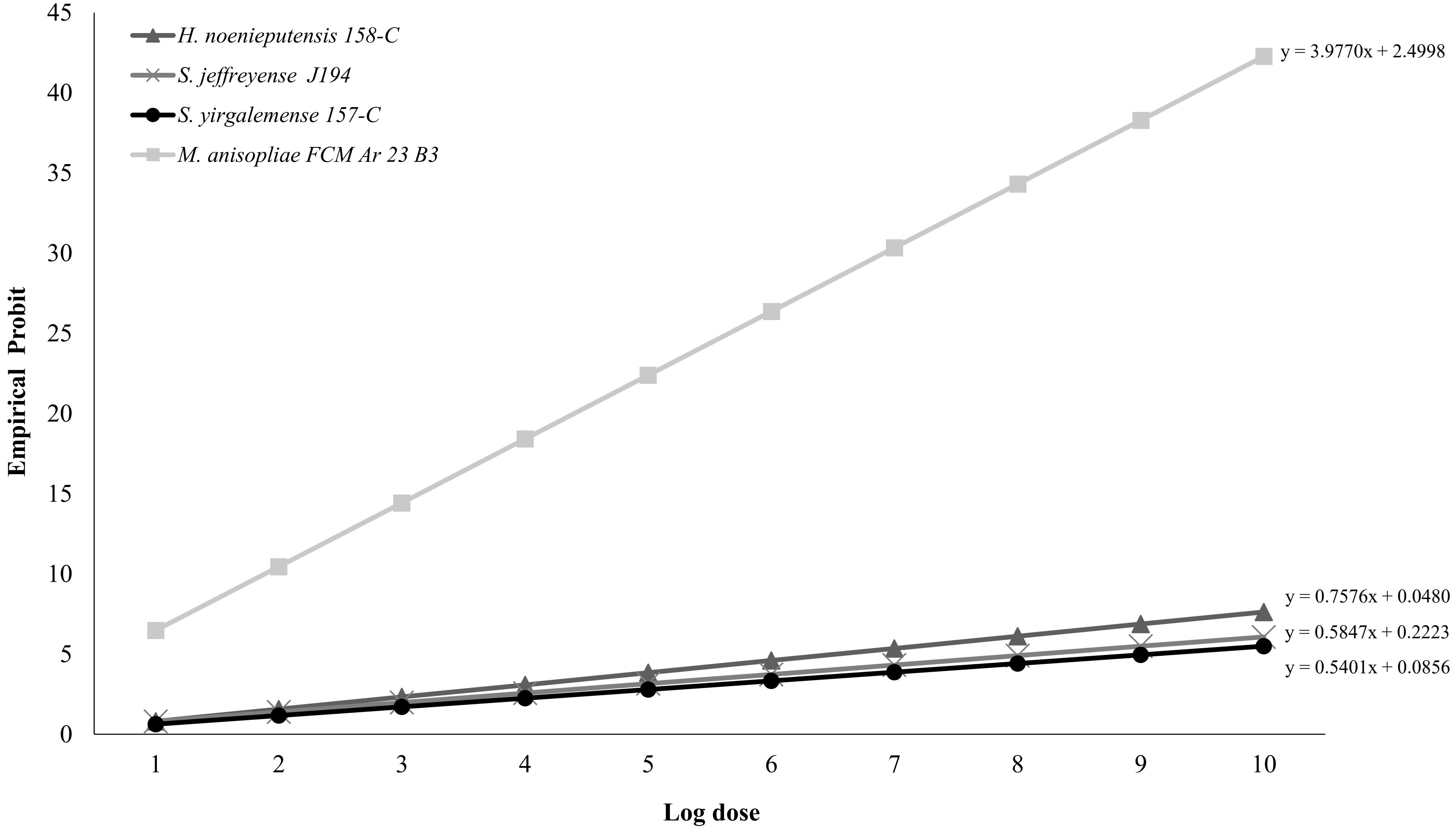


Figure 2.9. Dose-mortality Probit regression lines for fifth instar FCM treated with three EPN species *Steinernema yirgalemense* 157-C*, Steinernema jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C, and the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3.

The LC_{50} values for the three EPN species ranged between 4.38 IJs and 7.11 IJs, with *S. yirgalemense* and *S. jeffreyense* being the most and similarly virulent (Table 2.1). *Metarhizium anisopliae* FCM Ar 23 B3 recorded a LC_{50} of 3.42×10^5 conidia/ml. As expected, an increase in concentration was coupled with an increase in FCM mortality for all the tested agents (Figure 2.9).

Table 2.1. Lethal concentrations (LC_{50}, LC_{70} and LC_{90}) of three EPN species: *Steinernema yirgalemense* 157-C*, Steinernema jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C, and the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3, when applied to fifth instar FCM, obtained from a Probit analysis of dose response bioassays.

Control Agents	**LC_{50} (± SE)**	**LC_{70} (± SE)**	**LC_{90} (± SE)**
*H. noenieputensis**	7.11 (± 0.5)	14.02 (± 1)	38.6 (± 4.4)
*S. yirgalemense**	4.38 (± 0.5)	11.55 (± 1)	46.9 (± 7.7)
S. jeffreyense *	4.47 (± 0.5)	10.96 (± 0.9)	40.05 (± 5.8)
M. anisopliae **	3.42 (± 0.41)	14 (± 1.98)	121 (± 25.0)

* EPN units = IJs/larva

** EPF units = ($\times 10^5$) conidia/ml

2.4 DISCUSSION

All three EPN species tested in this study were shown to be virulent against fifth instar FCM and when applied at the recommended dose of 50 IJs per larva, larval mortality greater than 90% was recorded. This supports the results found by Malan et al. (2011) who conducted preliminary laboratory screening of *Steinernema yirgalemense* on three FCM life stages (fifth instar, pupae and emerging adults) and found the EPN to be virulent against all life stages. Malan et al. (2011) found that when 50 *S. yirgalemense* IJs were applied, 100% FCM fifth instar mortality was observed. The laboratory studies conducted by Malan et al. (2011) support the results in this study where 95.56% larval mortality was achieved. In addition, Steyn et al. (2019b), investigated the virulence of *S. yirgalemense* against FCM within the field and found when 30 IJs/cm^2 were applied, a mortality of 86% was achieved. Steyn et al. (2019a) also found that when *in vitro* reared *S. yirgalemense* were applied to the field > 60% of FCM larvae were infected after seven days. This suggested that *S. yirgalemense* could possibly be used as a control agent for FCM in citrus orchards in South Africa, due to the observed virulence. Although these studies did show EPN virulence towards FCM, a standardised study investigating all three EPN species on a particular FCM life stage (fifth instars) had not been conducted until now. This study found that when using a standardised bioassay protocol, *Steinernema yirgalemense* 157-C*, S. jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C, were able to induce an average percentage mortality of 95.56%, 93.33% and 91.11% respectively, with no significant difference. With regards to *S. jeffreyense,* studies by Steyn et al. (2019a) and De Waal et al. (2011) both achieved results similar to this study, which found that *in vitro* cultured *S. jeffreyense* induced 93.33% mortality in fifth instar FCM under laboratory conditions. *Steinernema jeffreyense* virulence was also tested against codling moth (*Cydia pomonella* L.) in laboratory studies by De Waal et al. (2011), who found that > 95% mortality was achieved on late instar larvae, suggesting that *S. jeffreyense* could be a useful MCA for other pests in South Africa.

Although only one strain from each EPN species was used in this experiment, strain variability has also shown to impact virulence. Fuxa et al. (1998) found that when testing three strains of *S. carpocapsae* against *Spodoptera frugiperda* (Lepidoptera: Noctuidae), a significant difference in virulence was recorded. Therefore, although *H. noenieputensis* showed a lower level of virulence (albeit not significantly different to the other two EPN species), it should not be ruled out as a potential control agent for FCM, because other strains may prove to be more pathogenic. These results only reflect laboratory findings; field responses may differ between species, hence, a lower laboratory efficacy (which is not significantly different to the other strains) should not warrant the elimination of this EPN strain. Having EPN species from both *Heterorhabditis* and *Steinernema* genera could be advantageous within IPM programmes, because of the variability amongst the genera, for example different feeding strategies symbiotic bacterial species associations and host immune responses.

The EPN genera characteristics also play a role in influencing variability in virulence. In this case the two *Steinernema* species performed better than the *Heterorhabditis* species. This has been observed in research by Glazer et al. (1991). However, the reverse was observed, where the *Heterorhabditis* species was shown to be more virulent compared to its counterpart *S. glaseri* when applied to *Spodoptera littoralis* (Lepidoptera: Noctuidae). In this case, *H.*

noenieputensis was not significantly different to the EPF isolate. From personal observations, the harvesting process for *H. noenieputensis* also took longer than the other two EPN species. This might suggest that this particular strain of *H. noenieputensis* is slower acting when infecting and causing mortality in FCM fifth instars. This may explain why no significant difference was recorded between this EPN and the EPF, which by nature have been characterised as slower acting (Hesketh et al. 2010).

Host invasion, evasion and extracellular enzymes are other factors that influence EPN pathogenicity and thus could account for the variations in LC values observed. Invasion of the host requires the EPN IJs to penetrate the host. Host location and method of penetration differs between the genera (Ishibashi & Kondo 1990). For example, *S. carpocapsae* can penetrate the host via the mouth, anus or spiracles (Triggiani & Poinar 1976), whereas *Heterorhabditis* spp. IJs use their characteristic "tooth" to penetrate the host's intersegmental membrane, as well as entering through natural host openings (Bedding & Molyneux 1982). Although methods for host penetration may not be a direct cause for increased virulence, the more aggressive nature of *Steinernema* spp. may explain why both *Steinernema* spp. used in this study had a greater virulence against FCM fifth instars than the *Heterorhabditis* species.

Once the host has been penetrated by the IJs and they have entered the haemocoel, the IJs' ability to develop, mature and reproduce also influences their virulence and therefore success as control agents (Simões & Rosa 1996). Evading the host's defences is fundamental for the success of the EPN within the host and is another determining factor for EPN virulence. The EPN need to successfully destroy the host's antibacterial factors to ensure a favourable environment has been created for their symbiotic bacteria (Simões & Rosa 1996). Their symbionts are also responsible for the release of extracellular enzymes, evading the host's defences and for tissue breakdown in the host. However, the pathogenic process is not fully understood with regards to both EPN and bacterial host evasion (Simões & Rosa 1996). The evasion process might be responsible for the reduced virulence observed in *H. noenieputensis* compared to the other two *Steinernema* species. The type of bacteria associated with the EPN are genera specific, with *Steinernema* and *Heterorhabditis* associated with *Xenorhabdus* spp. and *Photorhabdus* spp., respectively. These genera have shown a high degree of variability amongst different strains when pathogenicity bioassays were conducted (Simões & Rosa

1996). Both the bacteria genera and/or species and strains could account for a certain degree of discrepancies observed in this study. Each of the three EPN species were associated with different bacterial symbionts; *Steinernema yirgalemense* is associated with *X. indica* (Ferreira et al. 2016a), *S. jeffreyense* is associated with *X. khoisana* (Dunn et al. 2019) and *H. noenieputensis* is associated with *P. luminescens* (Malan et al. 2011). Because two of the EPN and their bacteria belonged to the same genera, this could also explain why the two had similar LC values that differed from the *Heterorhabditis* species.

Moving forward, these results have shown that level of virulence should not be factor when and if a decision needs to be made to select one species over another, regardless of the minor differences recorded. Instead, other factors should be considered such as, the environmental hardiness of the species/strains and the mass production and formulation protocols and cost effectiveness of these protocols. Both *S. yirgalemense* and *S. jeffreyense* are produced *in vitro* at Stellenbosch University, South Africa to date (Dunn et al. 2019; Ferreira et al. 2016b).

However, *H. noenieputensis* has no *in vitro* production protocol and still requires *in vivo* cultures, this is mostly attributed to challenges faced in culturing the symbiotic bacteria, *Photorhabdus luminescens* (Ferreira et al. 2013). Hence, investing into researching only the two *Steinernema* species as possible control agents for soil-dwelling FCM life stages, might be more beneficial from a formulation and virulence perspective. Another important factor to consider is the success of these species on other soil-dwelling life stages of FCM (Adom et al. 2020). For example, research on their effectiveness with pupae and emerging adults, which may also come into contact with the EPN during the eclosion process. Malan et al. (2011) and Steyn et al. (2019b) have already shown that *S. yirgalemense* has the ability to infect the FCM fifth instars, pupae and emerging adults. This should be taken further with the use of a uniform protocol for all three EPN species used in this experiment, due to their promising virulence results.

Although no significant difference was recorded between the three EPN species, there was a significant difference between the virulence of the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3 and *Steinernema yirgalemense* and *S. jeffreyense.* This is not unusual for several reasons. Firstly, the speed of kill of EPN is more rapid (48 h) than the 3-7 days for the most virulent EPF isolates (Hesketh et al. 2010). Secondly, EPN are still effective at low IJ concentrations, because a single IJ is able to induce host death (A.P. Malan, pers. comms). Lastly, although a single EPF conidium can penetrate the host cuticle, EPF are far more effective at high conidial concentrations (discussed in Chapter 1) (Chandler 2017; Hesketh et al. 2010). There was, however, no significant difference between the mortalities induced by the EPN species *H. noenieputensis* and *M. anisopliae*. The fungus was able to induce 80% mortality in fifth instar FCM when applied at 1×10^7 conidia/ml. This might have occurred due to the slower acting nature of *H. noenieputensis* and overall variation of EPN species/strains virulence.

The virulence of the EPF isolate *Metarhizium anisopliae* FCM Ar 23 B3 against FCM life stages and tolerance to environmental factors has been extensively studied in previous research by Goble et al. (2011), Coombes et al. (2015, 2016) and Acheampong et al. (2020a, b). In particular, the LC_{50} and LC_{90} values were determined by Coombes et al. (2015) and were found to be 1.92×10^6 and 1.67×10^8 conidia/ml, respectively. The percentage mortality of fifth instar FCM, when rescreened by Coombes (2013) based on the results from Goble et al. (2011), was found to be between 80 and 85%. These findings, although similar, cannot be compared directly to the results from this study due to the different methodologies used. Coombes (2013) and Goble et al. (2011) used sand bioassays where the EPF suspensions were applied directly to the sand before placing the FCM larvae into the container. However, this study used 24-well bioassay plates lined with filter paper and direct contact between the fifth instar FCM larvae and the conidial suspension was achieved by dipping the larvae into the suspension. The 24-well plate bioassays ensure conidial-larval contact is achieved, with a higher probability of conidia attaching to the host cuticle, whereas the sand bioassays, have a lower probability of conidial attachment to the host cuticle. However, the sand bioassays are more reflective of the natural environment in which the FCM fifth instar will move through the soil, where they will encounter the conidia. However, establishing mortality using the methodology in this thesis was important to allow for EPF-EPN interaction studies to be carried out effectively. Despite the different methodologies, this study did find that the mean percentage mortality of *M. anisopliae* FCM Ar 23 B3 was 80%, which corresponds with the findings and rescreening results by Coombes (2013) and Goble et al. (2011). With regards to the lethal concentration values, this study found a lower LC_{50} value (3.42×10^5 conidia/ml) compared to the higher concentration found by Coombes (2013) (1.92×10^6 conidia/ml). This variation in LC values (which is also applicable to the LC_{90} values) is most likely attributed to the difference in methodologies. Nevertheless, this new LC_{50} value can be used to compare the EPF isolate to other biological control agents that would require a similar 24-well plate bioassay protocol. However, if a positive (synergistic) interaction is found between the EPF and the EPN, then a more environmentally realistic protocol using the sand bioassays should be considered to determine how soil impacts the interactions between the MCA.

In conclusion, all three EPN species and the EPF isolate were successfully shown to be virulent against fifth instar FCM in uniform bioassay protocols. This study was able to determine the lethal concentration values (LC_{50}, LC_{70} and LC_{90}) for all four MCA, which can be used in future laboratory studies. Soil-bioassays, which are used more frequently in other studies were not the chosen bioassay methodology used to determine the LC values for the EPF in this study. Instead, bioassays using 24-well plates lined with filter paper discs were conducted, so that the methodologies for the EPF and EPN were the same. This was necessary for the combination bioassays in Chapter 3, so that the EPF and EPN could be applied using the same bioassay conditions. Lastly, the lethal concentration values (LC_{50}, LC_{70} and LC_{90}) are necessary for the combination bioassays that are reported in Chapter 3. A lower dosage of both the EPF and the EPN from the recommended rates was required to assess the type of relationship that occurs between the EPF and the three EPN species.

Chapter 3

EPF AND EPN INTERACTIONS FOR THE CONTROL OF FCM

3.1 INTRODUCTION

Biological interactions vary considerably (Ferrisa et al. 2001; Scheu 2002). The most well-known method for visualising these interactions is through food webs and trophic level interactions (Ferrisa et al. 2001; Scheu 2002). However, the types of interactions that can occur between organisms is more extensive than the commonly known predator versus prey complexes or competition for resources. Instead, interactions can be examined more extensively, to the point where the specific interactions between a set of organisms in a food web can be examined (Ferrisa et al. 2001; Scheu 2002). For this thesis, focus was on the interaction effect of two entomopathogenic microbes, EPN and EPF, when simultaneously infecting or inhabiting their target host.

Interactions between entomopathogenic organisms such as fungi, bacteria, viruses and nematodes have been well studied. The types of interactions that exist between two insect pathogens are categorised as either antagonistic, additive or synergistic (Piggott et al. 2015). Antagonistic interactions are broken down into intra- and inter-specific competition as discussed in Chapter 1 (section 1.5) (Kaya & Koppenhöfer 2010). Antagonism between EPN and EPF is known, with both organisms capable of inhibiting each other's presence within the host, either via the production of antibiotics (EPF inhibiting) or mycotoxins (EPN inhibiting) (Babercheck & Kaya 1990; Roberts 1981; Ansari et al. 2004; Wakil et al. 2017).

Additive and synergistic interactions are both characterised as positive or complementary interactions between biological organisms (Koppenhöfer & Kaya 1996). In these cases, both organisms are able to cohabit within the same host, working independently without inhibiting the other (Koppenhöfer & Kaya 1996). These interactions are attributed to the initial infector acting as a stressor to the host's immune system, which provides favourable conditions for secondary pathogens (Piggott et al. 2015). This favourable environment is brought about when the primary infector can overcome the host's defences, stressing the host. In turn, this allows the secondary infector to infect the host, with reduced defences to combat this secondary pathogen (Piggott et al. 2015). Additive or synergistic interactions are known to occur between entomopathogenic bacteria and EPN (Koppenhöfer & Kaya 1997; Thurston et al. 1993, 1994; Koppenhöfer et al. 1999), parasitoids and EPN (Shaik et al. 2020), chemicals and EPN (Koppenhöfer et al. 2000) and for the importance of this thesis, EPN and EPF (Barbercheck & Kaya 1991; Glare 1994; Choo et al. 2002; Wakil et al. 2017).

The type of interactions that might occur between multiple control agents is important information for determining the success, or failure of IPM programmes. Organisms found to be synergistic or additive could be applied simultaneously within the field to increase the control efforts by farmers. However, understanding antagonistic relationships is also important for IPM strategies because the application of organisms that inhibit other control agents should be avoided to ensure the IPM programme is cost and time effective. This thesis focuses on the interactions between three EPN species, *Steinernema yirgalemense* 157-C, *Steinernema jeffreyense* J194 and *Heterorhabditis noenieputensis* 158-C and a single EPF isolate, *Metarhizium anisopliae* FCM Ar 23 B3 when applied to fifth instar FCM. The aim of this chapter is to conduct combined bioassays to determine the type of interaction (antagonistic, additive or synergistic) between the EPF isolate and the three EPN species. Understanding these relationships can provide vital information that can be used in IPM programmes for the soil-dwelling life stages of FCM, with the emphasis on creating cost effective programmes.

3.2 METHODS AND MATERIALS

3.2.1 Insect cultures

FCM fifth instars used in these experiments were obtained as outlined in Chapter 2 (section 2.2.1).

3.2.2 Nematode cultures

The three EPN species assessed in the previous chapter, were used at their pre-determined LC_{50} concentrations. EPN were obtained and maintained as outlined in Chapter 2 (section 2.2.2.1).

3.2.3 Fungal culture

The EPF isolate assessed in the previous chapter was used at the pre-determined LC_{70} concentration (1.4×10^6 conidia/ml). The EPF culture was obtained and maintained as outlined in Chapter 2 (section 2.2.2.2).

3.2.4 Timing of EPN application

Previous studies have expressed the need for EPF inoculation to take place prior to EPN application, to ensure accurate establishment of interactions because the EPF is slower acting and requires more time to take effect (Piggott et al. 2015). However, the timing of the EPF application prior to EPN exposure is dependent on the EPF and EPN species. Therefore, it is important to determine the appropriate time for applying the EPN in this study. Another reason to validate for the time lag to be investigated, was that these experiments would take place under laboratory conditions, unlike most other studies, which used greenhouse conditions. Hence, the scale of the experiments would be highly reduced, however, the interactions would be highly magnified. Therefore, a pilot study was carried out to gauge the role that timing plays in this particular EPF and EPN interaction. All three EPN species *Steinernema yirgalemense, Steinernema jeffreyense* and *Heterorhabditis noenieputensis* were used in this experiment and applied to fifth instar FCM, previously inoculated with the EPF, *Metarhizium anisopliae* FCM Ar 23 B3.

This experiment used the recommended application rates for the EPF and the three EPN species, 1×10^7 conidia/ml and 50 IJs, respectively (A.P. Malan, pers. comm.). These suspensions were prepared as outlined in Chapter 2 (section 2.2.4). Five time periods were selected where the EPN species were applied 0 h, 24 h, 48 h, 72 h and 96 h post EPF application. Treatment of FCM fifth instars with these microbes in 24-well bioassay plates followed the same protocols as outlined in Chapter 2 (section 2.3.1). For all treatments, the fifth instar larvae were dipped into the fungal suspension at the start of the experiment. The EPN suspensions were then applied to the filter paper discs inside the bioassay wells at the different time intervals (i.e., 0, 24, 48, 72 and 96 h post-EPF application). The EPN application protocol differed to Chapter 2 (section 2.3.1) whereby, the EPN suspensions were now being applied to the filter paper discs inside wells that housed previously infected (EPF) fifth instar FCM. For each time interval (0 – 96 h) a control was added using 0.01% Tween® 20 acting as the fungal control, and distilled water as the EPN control, following the same time intervals as their EPN treatment counterparts. Each treatment consisted of five 24-well bioassay plates with 12 larvae per plate (total of 60 larvae per treatment), to restrict the movement of IJs into other wells. Once the larvae were dipped into the EPF suspensions, the bioassay plates were closed with glass covers and plastic lids, fastened by two elastic bands, placed into 2 L plastic containers and incubated in a CE room at 26ºC. Depending on the time interval the corresponding containers were removed as necessary and freshly prepared EPN suspensions were applied. The containers were then resealed as above and incubated as before.

Following the EPN protocol for assessing death, 48 h post EPN application, the corresponding containers were removed and the health of the FCM fifth instars was assessed and separated into four categories: 1) death by EPN, 2) death by EPF, 3) alive with necrotic spots, 4) alive with no signs of infection (EPF or EPN). For all categories, the larvae were separated and placed onto a tea sieve over a glass beaker, washed with distilled water and then treated as follows: (1) larvae succumbing to EPN infection were removed and placed into petri dishes lined with moistened filter paper and assessed as per Chapter 2 (section 2.2.4), (2) larvae succumbing to EPF infection were either recorded as mycosed, if sporulation had already occurred, or were surface sterilised in 70% ethanol and placed into petri dishes lined with moistened filter paper, until sporulation occurred, (3) larvae that were still, but which exhibited necrotic spots were placed into petri dishes lined with moistened filter paper until death occurred, such that EPF or EPN induced mortality could be determined as described in Chapter 2 (section 2.2.4) and (4) surviving larvae were placed into individual glass vials plugged with cotton wool and allowed to pupate. For the controls, the larvae were removed, washed, and placed into individual glass vials plugged with cotton wool and allowed to pupate. Any control deaths were as a result of handling error and never EPF or EPN infection. Dead control larvae were dissected to ensure no EPN were present, and no necrotic spots were present. After the

EPN infected larvae were dissected and the remaining EPF infected larvae sporulated, the percentage of larvae killed by either EPN or EPF were calculated.

3.2.5 EPF and EPN interactions

Combined EPF and EPN bioassays were conducted to determine the type of interaction that occurs between the EPF *Metarhizium anisopliae* FCM Ar 23 B3 and the three EPN species *Steinernema yirgalemense, Steinernema jeffreyense* and *Heterorhabditis noenieputensis*. Based on the findings from the pilot study (Results: 3.3.1) it was determined that a lower concentration was required to accurately determine the type of interaction. Hence, the dose response bioassays (Chapter 2) provided these lower concentrations in the form of the LC_{50} and LC_{70} for the EPN and EPF, respectively. It was also decided that all time intervals tested in the pilot study would be used with the lower concentrations, because they might play a role in influencing the results.

The experimental protocol followed the pilot study outlined in section 3.2.4, with the only differences being the concentrations used. In this case, the EPF LC_{70} value was used, 1.4×10^6 conidia/ml, and the LC_{50} values for all three EPN species were used: *Steinernema yirgalemense,* (4.38 IJs/larva) *S. jeffreyense* (4.47 IJs/larva) and *Heterorhabditis noenieputensis* (7.11 IJs/larva). The controls were the same as described in the pilot study (section 3.2.4) as well as the number of plates and larvae per time interval. The larvae were then examined once the experiments were completed and recorded as dead or alive and cause of death (EPF or EPN) as described above in section 3.2.4.

3.2.6 Statistical analysis

EPF and EPN interactions were analysed using the Chi-squared test (χ^2) in Microsoft Excel, Office 365. Each EPN species and its respective time interval were analysed individually using the methods outlined in Ansari et al. (2004). The observed value represented the average percentage of FCM fifth instars that died as a result of the combined EPF/EPN application (section 3.2.5). The expected value was calculated as: Expected mortality: $M_E = M_N + M_M (1 - M_N)$, where M_N and M_M are the observed proportional mortalities of FCM fifth instars infected by the EPN and the EPF alone (Ansari et al. 2004). Once the observed and expected values were determined for each of the EPN species and their respective time intervals, the values were placed in the Chi-squared formula:

$$\chi^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

Where: χ^2 = Chi-squared, O_i = Observed value, E_i = Expected value

The analysis of the χ^2 test results followed the protocol outlined in Ansari et al. (2004), Pelizza et al. (2015) and Finney (1964). The results from the Chi-squared test (i.e., the χ^2 value) were compared to the χ^2 table value of 1 degree of freedom. If the χ^2 value was less than the table value, the interaction was additive (Finney 1964; Ansari et al. 2004; Pelizza et al. 2015). Therefore, if the χ^2 value exceeded the table value, then the interaction was nonadditive (i.e., either synergistic or antagonistic) (Finney 1964; Ansari et al. 2004). To determine whether the interaction was synergistic or antagonistic, the following formula was used: $M_{MN} - M_E = D$. Where, M_{MN} = observed mortality (EPF and EPF combined application), M_E = expected value (as above) and D = difference. If D was positive the interaction was synergistic and if D was negative the interaction was antagonistic (Finney 1964; Ansari et al. 2004; Pelizza et al. 2015).

3.3 RESULTS

3.3.1 Timing of EPN application

When all three EPN species were applied to fifth instar FCM at the same time as *Metarhizium anisopliae* FCM Ar 23 B3 (0 h), no EPF deaths/infections were recorded, only EPN infections (Figure 3.1). When the EPN species were applied one day or more after the EPF were applied (24, 48, 72 or 96 h) EPF infections/deaths were observed (Figure 3.1). When the three EPN species were applied 72 h post EPF application ± 50% EPF/EPN mortality was observed (Figure 3.1). At 96 h after EPN application a slight difference was observed between the different EPN species, whereby *M. anisopliae* infections outweighed *H. noenieputensis* infections, however, for both *S. yirgalemense* and *S. jeffreyense* the ratio of EPF to EPN was still ± 50% (Figure 3.1).

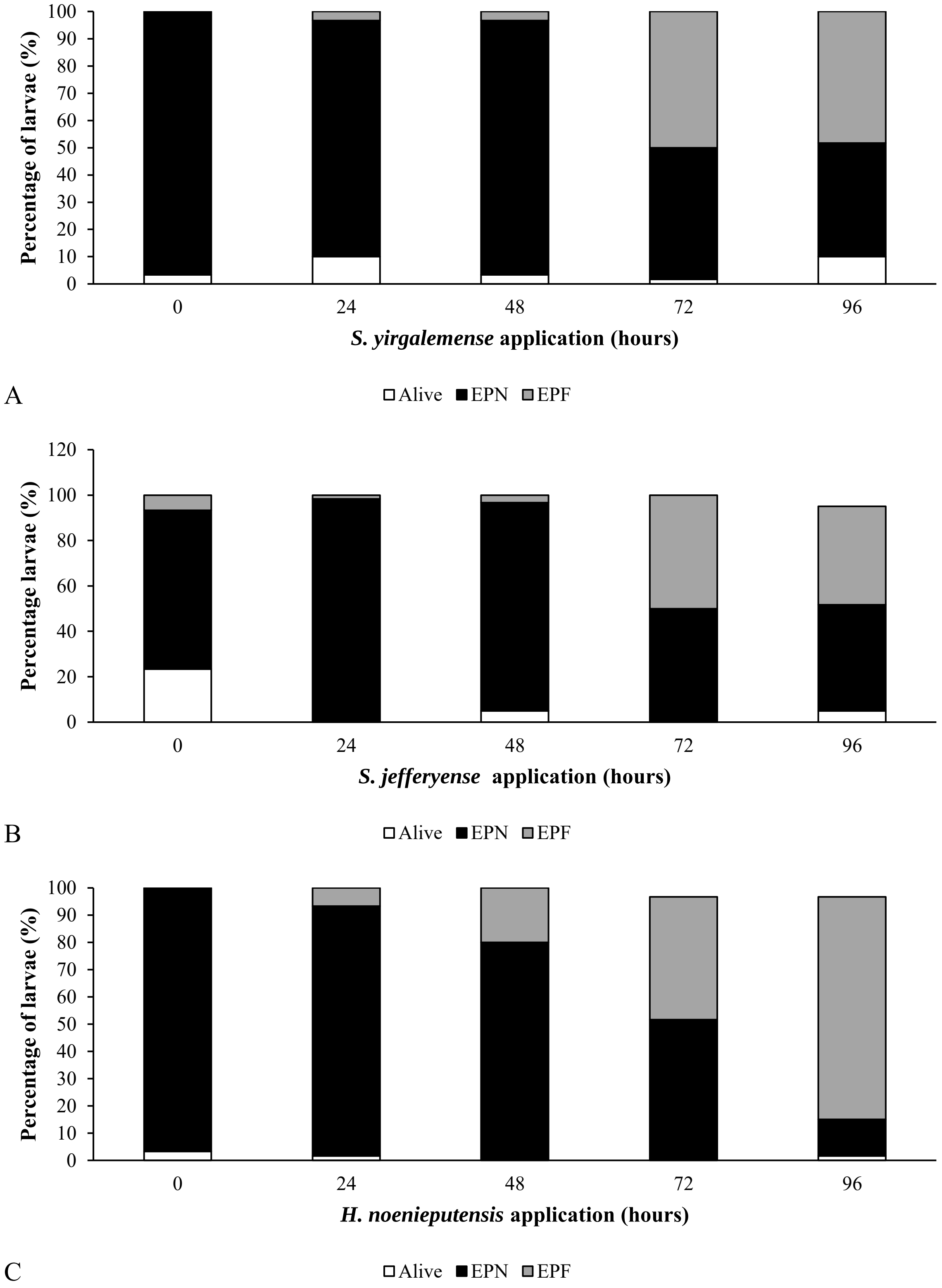


Figure 3.1. Percentage of larvae killed by A: *Steinernema yirgalemense* (top), B: *Steinernema jeffreyense* (middle) and C: *Heterorhabditis noenieputensis* (EPN) (bottom) or *Metarhizium anisopliae* FCM Ar 23 B3 (EPF). The EPF isolate was applied at 0 h thereafter, the EPN was applied at different time intervals (0, 24, 48, 72 and 96 h after EPF application).

3.3.2 EPF and EPN interactions

All three of the possible interactions that could have occurred were present (Table 3.1), one synergistic, twelve additive and two antagonistic. Specifically considering the individual EPN and the level of interaction that occurred when added to EPF infected FCM fifth instars, *Steinernema yirgalemense* was the only EPN species that showed a synergistic interaction at 0 h (i.e. EPF and EPN same day application) (Table 3.1). Additive interactions were observed from 0 – 96 h post EPN application for all EPN species. The exceptions to this trend were two antagonistic interactions observed when *S. jeffreyense* was applied at 24 h post EPF application and when *H. noenieputensi*s was applied simultaneously with the EPF (Table 3.1).

Table 3.1. Interactions recorded when combining entomopathogenic nematodes with *Metarhizium anisopliae* FCM Ar 23 B3 for the control of fifth instar FCM.

Treatments [a]	**Application rate IJs/larva** [b]	**Intervals (h)**	**Observed mortality** [c]	**Expected mortality** [d]	**χ^2** [e]	**Type of interaction** [f]
Sy	4.47	-	43.33	-	-	-
Sj	4.38	-	49.45	-	-	-
Hn	7.11	-	52.17	-	-	-
Ma	1.4×10^6	-	65.83	-	-	-
Sy + Ma	4.47	0	100.00	80.64	4.6	Synergistic
Sj + Ma	4.38	0	70.00	82.73	2.0	Additive
Hn + Ma	7.11	0	65.00	83.66	4.2	Antagonistic
Sy + Ma	4.47	24	83.33	80.64	0.1	Additive
Sj + Ma	4.47	24	46.67	82.73	15.9	Antagonistic
Hn + Ma	7.11	24	85.00	83.66	0.0	Additive
Sy + Ma	4.47	48	95.00	80.64	2.6	Additive
Sj + Ma	4.38	48	70.00	82.73	2.0	Additive
Hn + Ma	7.11	48	73.33	83.66	1.3	Additive
Sy + Ma	4.47	72	96.67	80.64	3.2	Additive
Sj + Ma	4.38	72	75.00	82.73	0.8	Additive
Hn + Ma	7.11	72	88.33	83.66	0.3	Additive
Sy + Ma	4.47	96	81.67	80.64	0.0	Additive
Sj + Ma	4.38	96	80.00	82.73	0.1	Additive
Hn + Ma	7.11	96	90.00	83.66	0.5	Additive

Table 3.1 continued:

[a] Sy = *Steinernema yirgalemense*, Sj = *Steinernema jeffreyense*, Hn = *Heterorhabditis noenieputensis*, + Ma = *Metarhizium anisopliae* FCM Ar 23 B3

[b] *Metarhizium anisopliae* FCM Ar 23 B3 was applied at the $_{LC70}$ first thereafter, EPN were applied in daily intervals.

[c] Percentage observed mortality of 60 FCM fifth instars.

[d] Expected mortality (Me) = Mn + Mm (1-Mn). Where Mn and Mm = proportion of observed mortality by EPN or EPF respectively when applied alone.

[e] Chi-squared value = $\chi^2 = \frac{(\text{Observed}-\text{Expected})^2}{\text{Expected}}$

[f] Interaction determined as follows: If (Observed – Expected) < 3.84 then Additive. If Observed > Expected, then Synergistic. If Observed < Expected, then antagonistic (Ansari et al. 2004; Pelizza et al. 2015)

3.4 DISCUSSION

The initial aim of this chapter was to determine the type of interactions that occur when a single EPF isolate is combined with three EPN species. These relations could have been antagonistic, additive or synergistic, with the latter interaction considered the most desirable for the overall improvement of control measures for the soil-dwelling fifth instar FCM. However, the identification of possible antagonistic interactions is also important, to ensure effective application of more than one control measure. This study found that all three types of interactions were recorded.

Steinernema yirgalemense was the only EPN species that showed a synergistic interaction at 0 h (i.e. EPF and EPN same day application) (Table 3.1). Additive interactions were observed from 0 – 96 h post EPN application for all EPN species. The exceptions to this trend were two antagonistic interactions observed when *S. jeffreyense* was applied at 24 h post EPF application and *H. noenieputensi*s was applied simultaneously with the EPF (Table 3.1). Additive interactions were the dominant type across all three EPN species. Exceptions to this were during the simultaneous applications of *Steinernema yirgalemense* and *H. noenieputensi*s which exhibited synergistic and antagonistic interactions, respectively. The only other antagonistic interaction observed was when *S. jeffreyense* was applied 24 h post EPF application. The specific interactions for the individual EPN species will be discussed further.

Heterorhabditis noenieputensis

Heterorhabditis noenieputensis has currently only been documented in South Africa (Malan et al. 2012; Stokwe 2016). Its symbiotic bacteria on the other hand has been extensively researched with particular attention given to its antimicrobial compounds and its compatibility with other organisms. Alone, *Photorhabdus luminescens* has been applied simultaneously with different fungal species (plant pathogens and EPF) and in most cases has been found to have a high antifungal ability (Chen et al. 1994; Jianxiong et al 1995; Shapiro-Ilan et al. 2014b). For example, when *P. luminescens* was applied with *M. anisopliae* (and three other EPF) an antagonistic relationship was found, based on the inhibition of conidial production and fungal growth (for all four EPF species), caused by the presence of the bacteria (Figure 3.2) (Ansari et al. 2005). Lalramchuani et al. (2020) also found an antagonistic interaction when *P.*

luminescens was combined with *Fusarium oxysporum* using a similar design as Ansari et al. (2005). They found that the bacteria exhibited a high antifungal ability causing 50-60% and 76-79% fungal inhibition at 48 and 96 h, respectively (Lalramchuani et al. 2020).

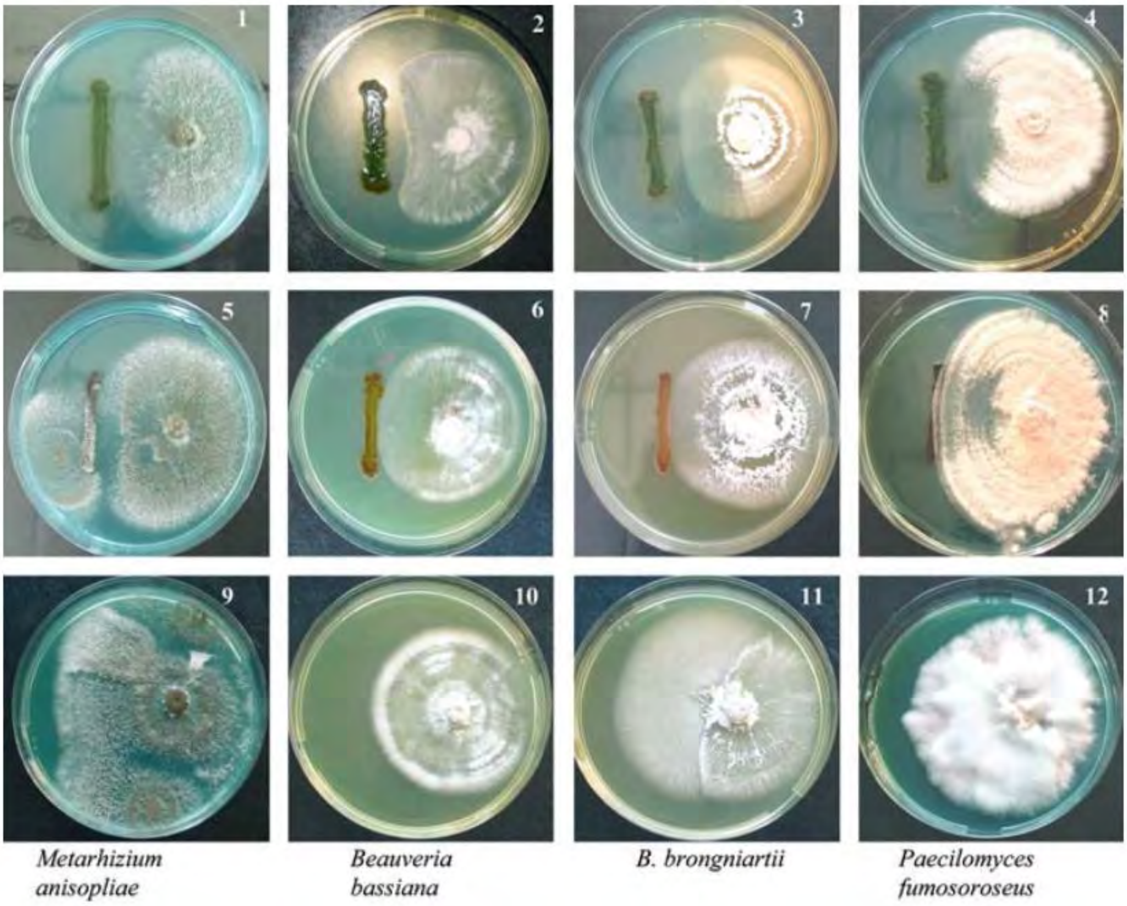


Figure 3.2. Antifungal activity from *Photorhabdus luminescens* (plates 1, 2, 3, and 4) and *Xenorhabdus poinarii* (plates 5, 6, 7 and 8) against four fungi on Nutrient bromothymol blue agar (NBTA) plates after 12 days at 25 ± 1ºC. Control plates without bacteria (plates 9, 10, 11 and 12) (Taken from Ansari et al. 2005).

These findings differ considerably to this study, which found that the majority of *H. noenieputensis* interactions observed were additive (except for the simultaneous application, which was antagonistic). A possible reason for this variation may be the *P. luminescens* strain exhibiting low levels of antifungal producing compounds. Alternatively, the *H. noenieputensis* strain used in this study, may have been causative, as it has been found to be slower acting with regards to infection and reproduction/harvesting from the hosts (A.P. Malan, pers. comm.). The slow infection time could be the reason for the additive interaction taking place because the fungus was allowed to establish within the host before antifungal compounds were produced by the bacteria, which may explain the lack of antagonistic interactions here compared to that observed in other studies.

Steinernema yirgalemense

Unlike *H. noenieputensis*, *Steinernema yirgalemense* and its associated bacteria, *Xenorhabdus indica*, have both been well documented, due to their global distribution. A range of interactions have been observed when *S. yirgalemense* was combined with different fungal species/isolates against a variety of insect hosts. A summary of some of these interactions can be found in Devi (2019). Specifically looking at the examples outlined in Devi (2019), that involve *S. yirgalemense* and *M. anisopliae* (Koppenhöfer & Kaya 1997; Thurston et al. 1993), antagonistic interactions were observed when applied to *Eriosoma lanigerum* Haussmann (Hemiptera: Aphididae). However, Anbesse et al. (2008) found both synergistic and additive interactions when *S. yirgalemense* and *M. anisopliae* were applied simultaneously on *Coptognathus curtipennis* Faimaire (Coleoptera: Scarabaeidae). When applied sequentially (EPN applied three weeks post EPF application) a stronger synergistic interaction was observed (Anbesse et al. 2008). Several studies have found varying interactions taking place between the EPN/bacteria and *M. anisopliae* (Koppenhöfer & Kaya 1997; Thurston et al. 1993; Anbesse et al. 2008). This is most likely due to the differences in EPF, EPN and bacterial strains and the different target insect hosts (Koppenhöfer & Kaya 1997; Ansari et al. 2004, 2006; Shapiro-Ilan et al. 2004; Devi 2019).

This study reported additive interactions as the dominant type across all three EPN, except for one interaction per EPN timed application. Additive interactions are characterised as two individual agents working independently from one another within the same host, which leads to an additive or complementary control effort (Kaya & Koppenhöfer 1996). This outcome may suggest that either the antifungal compounds produced by this strain of *X. indica* are not as effective at inhibiting this particular strain of *M. anisopliae* within the host, allowing the two to tolerate one another or, the EPF isolate was also producing low levels of mycotoxins that ensured its presence within the host was not impacted by the bacteria. The ability of *M. anisopliae* to inhibit bacterial growth has been documented. For example, Ansari et al. (2005)

showed that a crude extract of *M. anisopliae* CLO 53, when applied at the highest rate tested, inhibited two EPN associated bacteria*, P. luminescens* and *X. poinarii* (Figure 3.3).

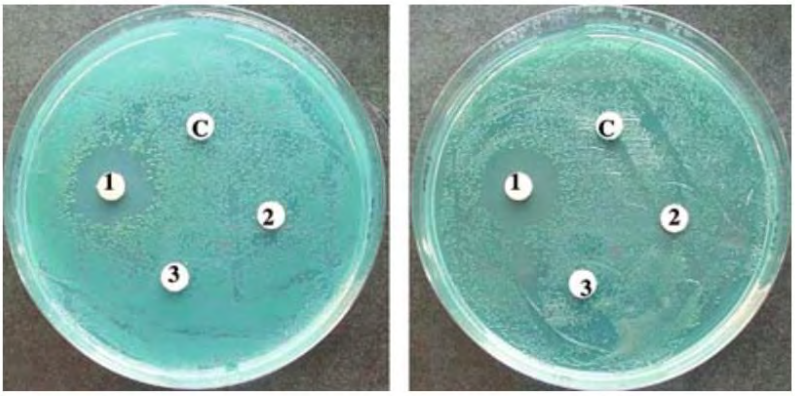


Figure 3.3. The effect of *M. anisopliae* CLO 53 crude extract on *Photorhabdus luminescens* (left) and *Xenorhabdus poinarii* (right) on NBTA plates. (1 = 1000 µl, 2 = 100 µl, 3 = 10 µl and C = control) (Taken from Ansari et al. 2005).

Steinernema jeffreyense

Like the other two EPN species, *S. jeffreyense,* exhibited additive interactions, barring the 24 h timed application, which resulted in an antagonistic interaction. These findings can be attributed to the same reasoning for additive interactions discussed above, whereby the two agents work independently from one another in a complementary manner (Kaya & Koppenhöfer 1996). *Steinernema jeffreyense* and its symbiotic bacteria *X. khoisana* has not been found elsewhere in the world, besides South Africa. Investigations into possible combined application of this EPN and its associated bacteria with other control agents has yet to be conducted in other studies. However, a study by Dreyer et al. (2019) investigated the associated bacteria's ability to produce antimicrobial compounds. Although only one antagonistic interaction was observed (discussed below), Dreyer et al. (2019) reported that this bacterial species does produce antimicrobial inhibiting compounds, the specific types of which are outlined in Dreyer et al. (2019). Based on these findings, two assumptions could be made. Firstly, the antimicrobial compounds produced by the *X. khoisana* are not virulent enough to inhibit the EPF *M. anisopliae*. Secondly, these MCA could potentially be used in combination with one another for the control of fifth instar FCM.

An antagonistic interaction was observed when *S. jeffreyense* was applied 24 h post EPF application. This was the only non-additive interaction that was observed between the 24 – 96 h timed applications. This result was not expected and does not correspond with the literature, which states interactions become more beneficial (additive or synergistic) when the timing between agents is increased (Ansari et al. 2004; Correa-Cuadros et al. 2016; Ibrahim et al. 2019). Instead, the most likely reason for this outcome is due to the lower than normal EPN deaths observed for the 24 h combined results. Therefore, the antagonistic interaction observed in this study between the EPF and *S. jeffreyense* should not be used as evidence to exclude this combination from further investigations into their interactions on a field trial level.

In conclusion, this study was the first to identify the interactions between these specific EPN and their associated bacteria when combined with *M. anisopliae* FCM Ar 23 B3. All three EPN species were found to have additive interactions when combined with the EPF and thus should be investigated further with particular focus on the bacterial and fungal interactions. The methods outlined by Ansari et al. (2005) and Lalramchuani et al. (2020) may be used as an initial screening for antagonistic or non-antagonistic interactions based on the EPF and bacterial combined interaction. These experiments can provide a second method of confirming these additive interactions.

Previous literature has shown that the types of interactions occurring between *M. anisopliae* and the two EPN *H. noenieputensis* (specifically the associated bacteria *P. luminescens*) and *S. yirgalemense*, vary considerably with all three types of interactions having been observed. This variation can be attributed to the types (species and isolate) of EPF, EPN and bacterial strains used in these experiments as well as the different host insects. Further investigations should also be carried out to assess the types of antimicrobial compounds released by both the bacteria and the fungi, for these specific strains (Koppenhöfer & Kaya 1997; Ansari et al. 2004, 2006; Shapiro-Ilan et al. 2004; Devi 2019). The third EPN, *S. jeffreyense*, and its symbiotic bacteria *X. khoisana* should be investigated further like the other two EPN species due to its promising virulence outlined in Chapter 2.

Chapter 4

GENERAL DISCUSSION

4.1 THESIS SYNTHESIS

This novel study investigated the types of interactions that occur between the fungal isolate *M. anisopliae* FCM Ar 23 B3 and three EPN isolates, *yirgalemense* 157-C*, S. jeffreyense* J194 and *H. noenieputensis* 158-C, with all microbial control agents (MCA) originating from South Africa. Three entomopathogenic nematodes (EPN) species and one entomopathogenic fungus (EPF) isolate had previously been selected as promising MCA for the soil-dwelling FCM life stages. This thesis focused on targeting the fifth instar FCM, which is the stage in the FCM life cycle that comes into contact with the soil environment, prior to pupation. Research has shown that the combination of two control agents can potentially increase the control efficacy of certain insect pests, when a synergistic interaction occurs between the two agents (Koppenhöfer & Grewal 2005).

The aims of this thesis were to firstly determine the virulence of the four MCA against fifth instar FCM, using a uniform methodology and then through the process of dose-response bioassays, determine the lethal concentrations of each of the control agents. Secondly, this thesis aimed to determine the types of interactions that occur between these specific EPN species when combined with the EPF isolate *M. anisopliae* FCM Ar 23 B3. This information can be used to improve IPM programmes for the control of soil-dwelling FCM life stages, which still lack commercially available control agents for field use. It should be noted that although a synergistic interaction was originally viewed as the most beneficial type of interaction for IPM programmes, antagonistic interactions can also provide useful information for successful implementation of an IPM programme. Specifically, antagonistic interactions can offer information on when not to apply agents simultaneously in the orchards.

The initial virulence screening reaffirmed the virulence of the four MCA against fifth instar FCM, when applied at the recommended doses of 50 IJs (EPN) and 1×10^7 conidia/ml (EPF).

The virulence of the three EPN species ranged between 91 and 96% mortality, with *S. yirgalemense* inducing the greatest FCM mortality. The EPF isolate induced 80% mortality in fifth instar FCM. The two EPN species, *S. yirgalemense* 157-C and *S. jeffreyense* J194 were found to be significantly different from the EPF isolate, however no differences occurred between the three EPN species, and *H. noenieputensis* and the EPF. The lethal concentration values for all four MCA were determined. The LC_{50} results showed that *H. noenieputensis* required a higher concentration of IJs (7.11 IJs) compared to the other two EPN species, 4.38 IJs (*S. yirgalemense*) and 4.47 IJs (*S. jeffreyense*). The LC_{50} value for the EPF isolate, *M. anisopliae* FCM Ar 23 B3 was recorded as 3.42×10^5 conidia/ml using the protocol established in this study.

The combined EPF and EPN application studies were able to determine the types of interactions that occurred between the MCA at both simultaneous and sequential EPN application. Additive interactions were the most dominant when *S. yirgalemense* and *H. noenieputensi*s were applied to FCM larvae that had previously been exposed to *M. anisopliae* 24-96 h prior to EPN application. *Steinernema yirgalemense* and *H. noenieputensi*s exhibited synergistic and antagonistic interactions, respectively when applied simultaneously with the EPF. *Steinernema jeffreyense* also exhibited additive interactions when applied to FCM fifth instars that had previously been exposed to *M. anisopliae* at 0 and 48-96 h post fungal application. Fungal application 24 h before EPN application resulted in an antagonistic interaction. However, it should be noted that this interaction was brought about by a substantial drop in EPN related deaths. This may have possibly skewed the results. An additive interaction is most likely the 'correct' interaction for this time period, based on the additive trend for all other time intervals.

4.2 INTERACTIVE EFFECTS

Determining the types of interactions (additive, synergistic or antagonistic) that occur between insect pathogens is an important step when developing multiple control methods for pest management. This is because the way these organisms interact within the shared environment, and more importantly within the same host species, can influence whether the control efficacy is enhanced or hindered because of the type of interaction taking place. This study was able to determine the types of interactions that occurred between the EPF isolate *M. anisopliae* and three EPN species. All three types of interactions were observed, and this variability in results is not uncommon when investigating entomopathogen interactions for the purpose of biological control (Devi 2019; Wakil et al. 2017). There are four major attributes that are believed to directly influence the type of interactions that occur: timing, the stress effect, host species and age, MCA species and isolate (including symbiotic bacteria and EPN). These hypothesised reasons may allude to why the types of interactions obtained from this thesis were brought about. Each interaction will be discussed below with reference to the attributes thought to bring about such results.

4.2.1 Additive interactions

Overall, this study recorded additive interactions to be the dominant type over synergistic or antagonistic interactions, with all three EPN species exhibiting additive interactions when applied sequentially post EPF application. Additive effects occur when two pathogens are combined but work independently from one another inside the host (Koppenhöfer & Grewal 2005). Hence, all three EPN species were acting independently from the EPF once they inhabited the fifth instar FCM hosts (except for simultaneous application of *S. yirgalemense* and *H. noenieputensis*, and 24 h post EPF application for *S. jeffreyense*). Additive interactions observed in this study could then suggest that the types of EPN (and symbiotic bacteria) isolates and the EPF isolate do not compete with one another hence, no antibiosis occurs (Tarasco et al. 2011). However, additive interactions may also be brought about when the timing of the EPF and EPN are not optimised, hence a possible synergistic interaction is underutilised or unknown.

The majority of studies investigating the interactions between MCA, stress the importance of timing, specifically the necessity for delaying the timing between the first and second agent (Koppenhöfer & Kaya 1993; Babercheck & Kaya 1991; Ansari et al. 2004; Wakil et al. 2017). Timing is an important factor because of the ‘stressor effect’ (Steinhaus & Martignoni 1970). It is believed that when two pathogens infect a host, the primary infector acts as the stressor and is responsible for the faster infection rate of the second pathogen. This is because the stressor has debilitating effects on the host (Piggott et al. 2015; Wakil et al. 2017). For example, Ansari et al. (2004) found that when *M. anisopliae* CLO 53 was applied first, it acted as a stressor by reducing host feeding. This in turn disturbed the behavioural, physiological and morphological characteristics of the host *Hoplia philanthus* Füessly (Coleoptera: Scarabaeidae) (Gaugler et al. 1994; Wang et al. 1995), ultimately causing the host to become more susceptible to secondary infections from other pathogens such as the EPN *H. megidis* and *S. glaseri*. When applied to the already fungus infected host, an additive result was found. Another study also investigating the potential interactions between EPN and EPF was conducted by Babercheck & Kaya (1991). The authors found that additive interactions were achieved when *H. bacteriophora* was applied to beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), that had previously been infected with *B. bassiana.*

This 'stressor' effect has also been shown to occur with other pathogen combinations. Koppenhöfer & Kaya (1997) found that when the bacteria, *Bacillus thuringiensis* subspecies *japonensis* Buibui strain (*Btj*) was applied to the white grubs, *Cyclocephala hirta* Leconte and *Cyclocephala pasadenae* Casey (Coleoptera: Scarabaeidae) at a minimum of seven days prior to EPN application (*H. bacteriophora and S. glaseri*), additive interactions were observed. This is because the bacteria act as a stressor, allowing for EPN infection to take place when host defences are diminished. The same trend was found when Thurston et al. (1993) applied *H. bacteriophora* to *C. hirta*, previously exposed to the milky spore disease bacterium, *Paenibacillus popilliae* (formerly *Bacillus popilliae*) Dutky. Increased insect mortality was observed. Host age has also been suggested as another factor that may influence the types of interactions that take place between MCA. Wakil et al. (2017) found that when combining *H. bacteriophora* with two EPF species, *B. bassiana* and *M. anisopliae*, younger instars of the RPW were more susceptible to the combination of EPF and EPN than older instars.

4.2.2 Synergistic interactions

Synergism is often viewed as the most favourable type of interaction for IPM purposes and is characterised as the combination of two control agents that are able to induce a control efficacy greater than the sum of the individual agents acting alone (Devi 2019). Like additive interactions, synergistic relationships are also shaped by factors such as application times, the stressor effect, host species, and more importantly the types of species/isolates intended to be used for combined application. In some instances, synergistic interactions can be brought about from interactions previously found to be additive. For example, Ansari et al. (2004) were able to induce a synergistic interaction by increasing the time between EPF and EPN application, reinforcing the importance that application timing may have on the interaction outcome. This trend of obtaining synergistic results by increasing the time between MCA exposure has also been noted in other studies (e.g. Correa-Cuadros et al. 2016; Ibrahim et al. 2019).

Although a single synergistic interaction was observed with the simultaneous application of *S. yirgalemense* and *M. anisopliae*, this was most likely not attributed to the reasons reported in previous literature, explaining how and why synergistic interactions occur. This is because the mortality results from the simultaneous application produced 100% (60 larvae) larval mortality due to EPN infection. This result was flawed because only an LC_{50} (30 larvae) was supposed to be observed, based on the concentration used. It is expected, based on the literature and the trend observed from *H. noenieputensis*, that an antagonistic interaction should have occurred, because simultaneous application has shown to produce antagonistic results, or (at best) additive results, but never synergistic. Because no EPF deaths were recorded at this stage, a synergistic interaction, which assumes that the mortality of two combined agents is greater than the sum of the individual agents, could not have taken place.

The ‘stressor’ effect is another reason why the ‘synergistic’ interaction was observed during the simultaneous application of *S. yirgalemense* and *M. anisopliae.* This is because the ‘stress’ effect is a vital component of synergistic interactions, because the weakened host (brought about due to the stressor agent) is more susceptible to a secondary infection, thus ultimately increasing the rate of mortality (Steinhaus & Martignoni 1970; Steinhaus 1958; Piggott et al. 2015; Devi 2019). The stress effect can be considered the most important factor for determining the type of interaction. Piggott et al. (2015) describe how synergism is brought about only when stressors operate in the same direction. However, when the stressors work in opposite directions, what is beneficial to one, is antagonistic to the other (Piggott et al. 2015). For example, Ibrahim et al. (2019) investigated the interaction that occurred between *B. bassiana* and *H. zealandica* on the last instar of the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae). Not only did they determine that the application of the stressor (EPF) first, resulted in a synergistic interaction, with 100% mortality observed, but they also investigated the host’s immune responses to the dual application. Ibrahim et al. (2019) were able to determine that the host’s immune responses changed when infected with both control agents, which suppressed two of the three immune responses investigated, phenoloxidase (PO) and detoxifying enzymes glutathione S-transferase (GST). Mehdi et al. (2018) was able to show that synergistic interactions can also occur across different instars, when combining *B. bassiana* with *H. bacteriophora* on RPW 2nd, 4th, and 6th instars. Younger instars are believed to be more susceptible to pathogens because they are still feeding and thus have a greater chance of getting infected by different MCA (Correa-Cuadros et al. 2016; Wakil et al. 2017).

The stressor effect has also been shown to occur when EPN are combined with other control measures, ultimately bringing about synergistic interactions. In Koppenhöfer et al. (2000) the relationship between the insecticide imidacloprid and two EPN species, *H. bacteriophora* and *S. glaseri*, was investigated in white grubs of the masked chafers *C. hirta* and *C. pasadenae*. They found that there was a strong synergistic relationship between *S. glaseri/H. bacteriophora* and the insecticide, which was promising for IPM strategies (Koppenhöfer et al. 2000). This relationship was attributed to the 'sluggish' behaviour of the insect as a result of insecticide exposure, which increased the chances of penetration of the host by the EPN species (Koppenhöfer et al. 2000). Two chemical insecticides can also have this effect, where the one acts as the stressor resulting in a greater control of the pest by the combined chemicals as opposed to the individual agents (Corbel et al. 2003).

The relationship between soil dwelling predators and above ground parasitoids has also been examined to determine whether combined agents can achieve greater control, with the parasitoids acting as the stressor. In Abdul Shaik et al. (2020) the relationship between *S. carpocap*sae and the parasitoid *Habrobracon hebetor* (Hymenoptera: Braconidae) on *Spodoptera littoralis* caterpillars was observed. They found that the venom of the adult parasitoid, injected into a host to suppress host mobility and defences, acted as a stressor, which increased the control efficacy when combined with the EPN *S. carpocapsae*, resulting in a synergistic relationship (Abdul Shaik et al. 2020). The EPN was found to be resistant to the venom and both the EPN and its symbiotic bacteria were able to reproduce in the presence of the venom (Abdul Shaik et al. 2020). The bacterium, *Bt* is a well-known biological control agent and its relationship with other control agents has been investigated. It was hypothesised that *Btj* acted as the stressor, which resulted in increased efficacy of the EPN species (Koppenhöfer & Kaya 1997).

The chance of obtaining a synergistic interaction, is heavily based on the species and isolates used for achieving control. Certain combinations can result in positive interactions, which leads to the synergistic outcome. However, other combinations can result in competition occurring between the two agents. With respect to MCA, antibiosis may occur, leading to antagonistic interactions. Therefore, determining the best combination of species/isolates for use in an IPM programme, is fundamental (Acevedo et al. 2007). One method for determining the types of combinations that are compatible and thus could lead to a synergistic interaction, is investigating the symbiotic bacteria and its compatibility with the EPN in question (Ansari et al. 2005; Correa-Cuadros et al. 2016; Lalramchuani et al. 2020). As previously discussed, (in Chapter 3) Ansari et al. (2005) tested the direct compatibility between the symbiotic bacteria and EPF isolate. Correa-Cuadros et al. (2016) also used similar methods to assess the interaction between the fungi *B. bassiana* Bb9205 and *M. anisopliae* Ma9236 and the bacteria *P. luminescens* (associated with *H. bacteriophora* HNI0100). This method of determining the correct combination of MCA is useful as a primary screening test because it investigates the organisms directly interacting within the host haemocoel (Tarasco et al. 2011**)**. In the case of EPF and EPN, it is in fact the bacteria that is responsible for bringing about host death through sepsis, and thus should be the initial screening step for compatibility with other MCA that also target the haemocoel.

Although synergistic interactions are brought about when the control efficacy of two agents is greater than the individuals alone, there are reports of this coming at a cost. Acevedo et al. (2007) reported that although synergism was achieved between *H. bacteriophora* JPM4 and *M. anisopliae* applied to sugarcane borer, *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae), it was at the expense of IJ production. This result was believed to be brought about due to the EPF and EPN isolates all exhibiting high levels of virulence. As such, Acevedo et al. (2007) recommended that a highly virulent EPN species/isolate should be combined with a low/moderate EPF species/isolate, to maintain the synergistic effect without the negative costs to reproduction and ultimately persistence in the environment.

4.2.3 Antagonistic interactions

Previous research has shown that it is most likely the symbiotic bacteria associated with the EPN that is in fact the cause for this negative interaction (Devi 2019). This is because once the EPN has entered the host and reached the haemocoel, the symbiotic bacteria are released, causing septicaemia within the host, and providing nutrients for the reproducing EPN (Devi 2019). However, the symbiotic bacteria also play a fundamental role in creating favourable environmental conditions within the host to ensure both the bacteria and EPN survival (Ishibashi & Kondo 1986). The bacteria are able to achieve this through the production of bioactive compounds, which exhibit antibacterial and antifungal properties (Ansari et al. 2005; Dreyer et al. 2018, 2019; Shapiro Ilan et al. 2004). These compounds are classified as an indirect form of antagonism, which is linked to both competition for resources and space (Kaya 2002; Kaya & Koppenhöfer 1996). Previous research has also investigated the specific relationship that occurs when the bacteria is plated on the same medium within a Petri dish as other microorganisms and found that a clear antagonistic interaction can be visually observed when examining the reduction in fungal growth (Ansari et al. 2005; Lalramchuani et al. 2020).

Antagonistic interactions are divided into two categories, firstly direct predation of the MCA by another organism and secondly competition between two MCA for the same resources (Roberts 1981; Singh & Faull 1988). For this thesis, it is the second antagonistic interaction brought about as a result of competition that will be discussed. When two MCA infect a single host, competition for resources (nutrients and space) can occur, this is brought about when either one or both MCA produce secondary compounds that cause debilitating effects on the other (Tarasco et al. 2011; Ansari et al. 2004; Shapiro-Ilan et al. 2004). Specifically, EPF and EPN can exhibit competitive characteristics through the production of antibiotics and antifungal compounds, respectively (Tarasco et al. 2011; Ansari et al. 2004; Shapiro-Ilan et al. 2004). However, it should be noted that the primary source of antifungal/antimicrobial compounds is the symbiotic bacteria associated with EPN (Tarasco et al. 2011; Ansari et al. 2004; Shapiro-Ilan et al. 2004). Like the additive and synergistic interactions, antagonistic relationships are highly dependent on the combination of species/isolate being used. Hence, it is imperative that the presence or severity of competition between potential agents be assessed to determine if the combined MCA are compatible.

Tarasco et al. (2011) were able to assess the level of antagonism between *Steinernema ichnusae* sp. n. bacteria (*Xenorhabdus bovienii*) and *B. bassiana*, using methods similar to those of Ansari et al. (2005), which have previously been discussed. They found that the growth of both organisms was inhibited and was attributed to both organisms trying to survive in the same environment (the insect haemocoel) (Tarasco et al. 2011). Several other studies have also found that the symbiotic bacteria are in fact antagonistic to EPF (Barbercheck & Kaya 1990; Chen et al. 1994; Shapiro-Ilan et al. 2004; Correa-Cuadros et al. 2016).

When the EPN *H. noenieputensis* 158-C was applied simultaneously with *M. anisopliae* FCM 23 B3, an antagonistic interaction was observed. This interaction was most likely brought about due to the antifungal compounds released by the symbiotic bacteria *P. luminescens*. Although *H. noenieputensis* has not been documented outside of South Africa, *P. luminescens* has been and still is well researched around the world. As an example, Lalramchuani et al. (2020) showed that after only 48 h the bacteria inhibited *Fusarium oxysporum* (Schlecht. emend. Snyder & Hansen) fungal growth by 50-60% and just under 80% after 96 h. Thus, the antagonistic interaction observed in this study could have been a result of the bacteria *P. luminescens* inhibiting fungal growth when applied simultaneously. Once the timing between the EPN and EPF applications had been increased (24-96 h) it is possible that the early establishment of *M. anisopliae* allowed the EPF to overcome the antifungal properties produced by the bacteria. As outlined above, additive and synergistic interactions are often only achieved when the stressor (in this case fungi) has been applied prior to EPN application (days or weeks). Hence, it is not surprising that an antagonistic interaction was observed when *H. noenieputensis* 158-C and *M. anisopliae* FCM AR 23 B3 were applied simultaneously.

Although synergistic interactions between baculoviruses and insecticides have been observed (Trang & Chaudhari 2000), there are studies that show that EPN combined with entomopathogenic viruses result in antagonistic interactions taking place (Kaya & Koppenhöfer 1996). For example, when *S. carpocapsae* was combined with a nucleopolyhedrovirus (NPV) (Bednarek 1986; Kaya & Burlando 1989) and a granulovirus (GV) (Kaya & Brayton 1978), the EPN development was inhibited. In this case the stressor imposed by the NPV, which weakened the host integument and disintegrated the host cuticle, negatively impacted developing EPN within the host. This resulted in their exposure to the environment, which led to their desiccation (Kaya & Koppenhöfer 1996). When the EPN was applied with the GV, a reduced number of progeny was exhibited as a result of less nutrients available for EPN development due to the GV infection (Kaya & Koppenhöfer 1996). Further investigations should be carried out to determine the relationship between different viruses and EPN and how timing of the combined applications could be improved to induce synergistic interactions, if at all. Sequential application with the stressor organism applied first, often results in additive or synergistic interactions. Therefore, simultaneous application, should have the opposite effect. Koppenhöfer & Kaya (1997) were able to show that during simultaneous application of *Btj* and *H. bacteriophora* and *S. glaseri*, antagonistic interactions were observed and when applied sequentially, the interactions were synergistic. The same results were found in previous studies by Babercheck & Kaya (1990) and Correa-Cuadros et al. (2016).

The environmental requirements of MCA have also shown to influence the types of interactions that occur between combined agents. Babercheck & Kaya (1990) have attributed factors such as temperature thresholds, moisture requirements and soil type, as reasons for why antagonistic or non-synergistic interactions are observed. They stated that the specific species/isolates are adapted to their natural climatic conditions, and if those conditions do not match up, then it is possible for the two control agents to never encounter one another in the same host. Babercheck & Kaya (1991) have also speculated that the health of the insect host may also determine the type of interaction when two MCA are combined. For example, if the secondary infector (e.g. EPN) prefers healthy hosts over previously infected (EPF) hosts, the EPN may choose not to invade sick hosts. Hence, there is no combined application occurring (i.e. no cohabitation of a single host) and only antagonistic interactions can occur because the sum of individual agents should not be more than the "combined" agents.

Although synergistic interactions are more desirable for IPM purposes, antagonistic results can also provide useful information about how insect pathogens and/or other control agents will interact within the environment. For example, if two commercially available agents are being used and have an overlapping application time range, understanding the type of interaction is imperative. If the two agents, although not applied for the purpose of combined application interact and yield a negative (antagonistic) interaction, a reduction in pest mortality may be experienced (Jaques & Morris 1981). A decrease in the persistence of the agents in the field may also occur as a result of antibiosis, which could lead to an additional pesticide application being necessary, which ultimately increases the cost of this control programme (Jaques & Morris 1981).

4.3 FUTURE RESEARCH AND RECOMMENDATIONS

Moving forward, the manner in which we investigate the types of interactions between EPF and EPN needs to be conducted uniformly. The ideal method for doing this would be to conduct a series of laboratory and field combination experiments. The first round of experiments should act as an initial screening test, whereby the type of interaction between the EPF isolate and the EPN symbiotic bacteria should be determined. As previously discussed, Ansari et al. (2005), along with other interaction studies, have outlined a method for conducting fungal/bacterial combination trials (Tarasco et al. 2011; Lalramchuani et al. 2020). These methods have also been followed to investigate new methods for controlling plant fungal pathogens, by investigating possible antagonistic interactions between EPN symbiotic bacteria through their production of antifungal properties (Wang et al. 2011; Hazir et al. 2016; Bock et al. 2014; Chacón-Orozco et al. 2020). Although these studies have focused on obtaining fungal control agents, as opposed to combined agents for insect control, the principal aim is the same: predicting the type of interaction between two microorganisms, by plating the two agents on the same agar source and assessing whether inhibition of the growth parameters arise. Lalramchuani et al. (2020) cites a formula from Balouiri (2016), whereby antifungal activity is assessed by measuring the diameter of the fungal growth when combined with the bacterial isolate against the control plate (fungus only). Once the initial screening of the bacterial and fungal isolates has been completed, promising isolate combinations (non-antagonistic) should be tested further, this time incorporating the EPN and the host insect.

In chapter two, the issue of varying laboratory methodologies was discussed. This study chose to investigate the types of interactions between the EPF isolate and the three EPN isolates, using only 24-well bioassay plates. Hence, this interaction was purely an assessment of the compatibility of the agents and not a true representation of how these agents will act in their natural environment, the soil. Although the methodology selected for this thesis provided substantial results, if the previous experiment (bacterial and fungal compatibility) is initially carried out, the EPN and EPF trials using hosts could be conducted using soil bioassays immediately as the initial interaction has already been predicted. Soil assays in the laboratory have made use of soil in cups, with specific soil requirements been carried out (type, nutrients, water potential) (Babercheck & Kaya 1991; Ansari et al. 2004; Ibrahim et al. 2019; Shapiro-Ilan et al. 2004). Choo et al. (2002) also determined that the soil and substrate used has a direct impact on the types of interactions that occur. This type of experimental setup is also more reflective of how the pathogens will interact with the target species, as opposed to the insects being dipped directly into the conidial suspension, as occurred in this study. The movement of the larvae over soil previously applied with the fungal suspension is a better representation of field conditions.

Potential EPF and EPN combinations found to exhibit high levels of additivity or synergism in laboratory bioassays, should be considered for field trials. Because EPN species under early examination as potential control agents are usually not in a formulated suspension, greenhouse experiments are one method of overcoming certain detrimental environmental conditions that could negatively affect the unprotected EPN, while still obtaining field-like conditions. Ansari et al. (2004) commenced with greenhouse experiments on unformulated *M. anisopliae* CLO 53 and *H. megidis* and *S. glaseri* against third-instar *H. philanthus* once laboratory soil bioassays had led to additive and synergistic interactions. Choo et al. (2002) and Ansari et al. (2004) did make use of formulated MCA and thus were able to apply the EPN/EPF isolates in the field where the pest species occurred, to assess whether the interactions obtained from their laboratory studies were altered as a result of field conditions and whether the combination of two agents was more effective at inducing insect mortality than the agents acting alone.

Utilising these methodologies can streamline future interaction assessments for EPF and EPN isolates for control of FCM and other insect pests. The use of laboratory and field trials will provide vital information as to whether the combination of two agents is better than a single agent acting alone so that future IPM programmes can be improved to achieve greater control efficacies against a particular pest. If the results show that two agents bring about the same level of control as that achieved by a single agent, this information is equally important for IPM programmes for several reasons. Firstly, funding research on and development of a single agent is a lot cheaper and less time consuming than researching two, and this could allow the individual control agent to be studied in greater depth. Secondly, although synergism might be observed, a reduction in progeny production and MCA development might still occur when two agents are combined. Hence, the persistence of the two agents in the field might be reduced, ultimately resulting in necessary follow up applications. However, if a single agent is released, with similar control efficacies as the two combined agents, it might persist longer in the field because it is not competing with other agents and thus fewer follow up applications need to be carried out.

Lastly, the FCM IPM programme in South Africa relies on a multitude of control techniques and control agents and chemicals (Moore 2021). The application of these controls may overlap throughout the season, and although they might not be used to target the same life stage (or pest species) (Moore 2019, 2021), the agents may come into contact with one another. These indirect interactions could have no impact on the persistence of the agents. However, if shown that certain combinations or a particular control agent has sublethal or antagonistic effects on other control agents, the persistence of agents could be hindered. Hence, fully understanding how different control agents, interact with one another is an essential component in improving IPM programmes, regardless of whether these agents were intended for combined application or not.

4.4 CONCLUSION

In conclusion, the combined application of the EPF isolate *M. anisopliae* FCM AR 23 B3 and three EPN isolates: *S. yirgalemense* 157-C, *S. jeffreyense* J194 and *H. noenieputensis* 158-C, against fifth instar FCM resulted in mostly additive interactions when the EPN agents were applied 24 h post EPF application. Although no strongly synergistic interactions were observed, additive interactions have been shown to reach a synergistic level when certain parameters are changed (e.g. timing between application, soil types), and moving from laboratory to field trials. Hence, following the compatibility methodology outlined above, all three EPN species should be considered for laboratory soil bioassays, with the intention to conduct field trials. It is also highly recommended, due to the variability with the types of interactions observed between different isolates from the same species in other studies, that the symbiotic bacteria and the EPF isolate should be plated together to confirm that neither agent is causing significant inhibition of the other's growth. Regardless of the types of interactions that are obtained from field trials, all four MCA have exhibited a strong virulence against fifth instar FCM. Thus, these pathogens should continue to be considered as potential control agents for the soil-dwelling life stages of FCM, which still lack adequate control measures within citrus orchards in South Africa.

Chapter 5

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