Efficacy, host-finding ability and application methods of entomopathogenic nematodes to control pupae of *Gonipterus* sp. 2 (Coleoptera: Curculionidae)

By

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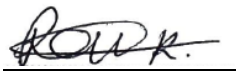
Declaration

I, Innocent Lephaswa Rakubu declare that the dissertation, which I hereby submit for the degree MSc in Entomology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

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Preface

Entomopathogenic nematodes (EPNs) from the families Steinernematidae and Heterorhabditidae are obligate parasites of many soil-dwelling insect pests. The *Steinernema* and *Heterorhabditis* nematodes have a symbiotic relationship with bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively. The nematodes penetrate the insect hosts using natural openings and kill the host, with the aid of their symbiont bacteria, 48h post-infection. Despite being listed as biological control agents for many insect pests in agriculture and horticulture, only a few pests of forest plantations are controlled with EPNs.

Gonipterus sp. 2 (Coleoptera: Curculionidae) is one of the important invasive pests of eucalypt plantations in South Africa and other countries. The adult beetles and larvae of this pest feed on the leaves of eucalypt trees, thus causing defoliation that results in poor tree growth. Chemical and biological control is used, but these are focused on the egg, larval and adult stages of the beetle. EPNs can potentially be incorporated into the management strategies of this pest to target the soil-inhabiting pupal stage.

This dissertation demonstrates the efficacy of five local South African EPN species, namely *Heterorhabditis neonieputensis*, *H. safricana*, *Steinernema fabii*, *S. jeffreyense*, and *S. yirgalemense* against pupae of *Gonipterus* sp. 2, and examines factors that can influence the efficacy of these EPNs, including host-finding strategy and application method. **Chapter 1** reviews the biotic and abiotic factors that hinder the full potential of EPNs as biological control agents in forestry and orchard plantations and suggests several ways to remediate these factors. The chapter further reviews the formulation techniques and application methods that facilitate the use of EPNs for controlling insect pests in forest plantations.

In **Chapter 2** the virulence of five local EPN species, namely *Heterorhabditis neonieputensis*, *H. safricana*, *Steinernema fabii*, *S. jeffreyense*, and *S. yirgalemense*, on the pupae of *Gonipterus*

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sp. 2 was assessed. 12-well bioassay plates were used as the infection arena. Ten wells of the 12-well bioassay plates received one pupa each and then inoculated with EPNs at a concentration of 200IJs/pupa. Each EPN species was inoculated on three bioassay plates each containing 10 pupae of *Gonipterus* sp. 2. Pupal mortality was recorded 48h post-inoculation. From the five EPN species, the most virulent species was selected to test for its lethal concentration (LC_{50} and LC_{90}). The effects of the pupal age of *Gonipterus* sp. 2 on the virulence of the selected EPN species were also determined.

EPNs locate their hosts by using either of three host-finding strategies, namely ambusher, cruiser, or intermediate foraging strategies. EPNs that use a cruiser foraging strategy actively seek out their host by following host-associated cues and are effective against sedentary hosts found deep in the soil. EPNs that use an ambusher foraging strategy use a sit-and-wait mechanism where they stand with tails so that they can attach to the mobile insect hosts passing within their striking range. The intermediate foraging nematodes possess characteristics of both ambush forager and cruise forager nematodes. Understanding the host-finding strategies of EPNs is important to inform which EPN species will be effective against a specific insect host and thus in **Chapter 3,** the host-finding strategies of the five local EPN species were investigated. This was done by observing the responsiveness of EPNs to the host's presence and their ability to locate and infect the host buried at a depth of 10 cm. To further understand the host-finding strategies, the distance travelled by EPNs on a substrate with two different textures (i.e. smooth texture and rough texture) was observed and compared. The observed behavioural patterns of EPNs in the study were used to infer the host-finding strategies.

The ease of EPN application using agricultural spray equipment and irrigation systems can influence the adoption and popularity of EPNs. These application methods vary in terms of efficacy as they affect the survival and dispersal of EPNs. In **Chapter 4**, the impact of two application methods, namely nematode-infected cadavers and an aqueous suspension, on the

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efficacy of *S. yirgalemense* was assessed. *Steinernema yirgalemense* was used because it had provided the highest pupal mortality against *Gonipterus* sp. 2 in **Chapter 2.** Pot trials where the target pupae of *Gonipterus* sp. 2 were buried at a depth of 10 cm were used, and pupal mortality between the treatments was compared.

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Summary

Gonipterus sp. 2 is a pest of economic importance to South Africa as it threatens the sustainability of eucalypt plantations. The adult beetles and larvae cause damage to *Eucalyptus* spp. through defoliation. There are currently no insecticides registered to control *Gonipterus* sp. 2 and only limited control is achieved by using an egg parasitoid, *Anaphes nitens*. It is thus necessary to explore other alternatives such as entomopathogenic nematodes (EPNs). Entomopathogenic nematodes of the families Steinernematidae and Heterorhabditidae have a symbiotic relationship with bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively. They are obligate parasites of many soil-dwelling insect pests of economic importance. EPNs are mass-produced for commercial purposes and distributed around the world. Despite the documented efficacy however, newly identified EPN species should be tested against economically important pests. In this study, the efficacy, host-finding ability and application methods of five local EPN species, namely *Heterorhabditis noenieputensis*, *H. safricana*, *Steinernema fabii*, *S. jeffreyense*, and *S. yirgalemense*, to control pupae of *Gonipterus* sp. 2 were investigated. Results from laboratory bioassays indicated that *S. yirgalemense* provided the highest pupal mortality and the lethal concentration of this EPN species was determined, namely LC_{50} = 45.7 IJs/pupa and LC_{90} = 150 IJs/pupa. *Steinernema yirgalemense* was also confirmed to kill pupae within their pupal cases and provided high mortality of the two pupal age groups tested, namely 1-3 days old and 9-12 days old. The host-foraging strategies of the five local EPN species were investigated by studying their movement behaviour in the presence of the host and comparing the distance they travel on a rough substrate and a smooth substrate. *Heterorhabditis noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense* were able to locate larvae of *G. mellonella* buried at a depth of 10 cm, thus causing high mortality. This suggested that the four EPN species use a cruiser foraging strategy, although this result was not supported when comparing the movement of those EPNs on the

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two substrates. *Steinernema fabii* was not effective at locating the larvae of *G. mellonella*, resulted in low larval mortality, and showed a reduced movement on a rough substrate, suggesting that it uses an ambusher foraging strategy. The infective juveniles of *S. yirgalemense* applied with both nematode-infected cadavers and aqueous suspension application methods were equally and highly effective at locating and infecting *Gonipterus* sp. 2 pupae buried at depths of 10 cm. This is the first study in South Africa to report the potential of five local EPN species as biological control agents of pupae of *Gonipterus* sp. 2.

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Chapter 1

Application of entomopathogenic nematodes for the control of underground insect pests of orchards and forest plantations

Abstract

Entomopathogenic nematodes (EPNs) have shown the potential as biological control agents for many important pests from various insect orders. The potential of EPNs to parasitize a large number of insect pests served as an advantage for them to be commercially marketable and globally distributed. The development of formulation technologies and the adoption of agronomic conventional liquid spray equipment and irrigation systems have facilitated the large-scale application of EPNs. However, various abiotic and biotic factors namely temperature, soil characteristics, host species, and foraging strategy can negatively affect the successful application of EPNs. Approaches such as modifying the soil environment, genetic improvement, use of adjuvants, and selection of genetically superior species can help to improve the survival of EPNs post-application. Other considerations to improve EPN efficacy include targeting the most susceptible developmental stages of the insect pest, using effective application rates of EPNs, and matching the host-finding strategy of EPNs to the targeted insect pest. The persistence of nematodes in the soil post-application is a key factor for achieving high pest suppression. Applying nematodes to soil conditions conducive to their survival and when susceptible developmental stages of the targeted insect pests are abundant can help prolong the persistence of EPNs. Extensive literature exists on the application of EPNs to control important insect pests of some crops but is scarce regarding the application of EPNs to control pests of orchards and plantation forests. This review discusses the methods used to apply EPNs to control soil-dwelling insect pests of orchards and forest plantations. We give

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special attention to factors affecting the survival of EPNs and approaches that can help to improve their survival and efficacy post-application.

Introduction

Chemical insecticides have had a major role in controlling insect pests (Dent and Binks 2020). However, the extensive use of chemical insecticides to manage insect pests has been associated with various unintended negative impacts (Gill and Garg 2014). For example, residues of chemical insecticides in the soil and on crops pollute the environment, reduce the number of beneficial insects, and threaten human health (Kumar et al. 2008; Ansari et al. 2014). In addition to the negative impacts of chemical insecticides, many insect pests have developed resistance to several chemical insecticides (Forgash 1984; Soderlund 1997). The negative impacts and development of insecticide resistance have elicited the need to consider biological control agents (natural enemies) as alternatives for pest management. Among the biological control agents considered for the management of soil pests, entomopathogenic nematodes (EPNs) have gained much attention, mainly because of their high virulence to a wide range of insect pests and ease of mass culture (Kerry and Hominick 2002; Katumanyane et al. 2018).

Entomopathogenic nematodes are soft-bodied non-segmented roundworms and naturally occurring obligate parasites of many soil-dwelling insects (Verma et al. 2020). EPNs belong to the phylum Nematoda and the families Steinernematidae and Heterorhabditidae (Stock and Goodrich-Blair 2012). *Steinernema* and *Heterorhabditis* nematodes have a mutualistic relationship with bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively (Poinar Jr 1990; Smart Jr 1995). They are among other invertebrate parasitic nematodes from families such as Allantonematidae, Mermithidae, Neotylenchidae, Sphaerularidae, and Rhabditidae, which have potential as biological control agents (Stock and Goodrich-Blair 2012). EPNs have been isolated from the soil and soil-dwelling insects and are distributed across all geographic

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regions, except in Antarctica (Abate et al. 2018). Ever since the potential of EPNs as biological control agents was noticed, their discovery has been increasing exponentially. As of 2020, about 100 steinernematid and 21 heterorhabditid nematodes were described globally (Bhat et al. 2020).

The techniques for large-scale production of EPNs in solid or liquid cultures developed in the 1980s, the development of formulation techniques, and the ease of application using spray equipment and irrigation systems, have facilitated the commercialization of EPNs (Ehlers 2007). Formulation techniques are used for storage, handling, and transportation; hence making it easy to commercialize EPNs globally. Attributes that encouraged the commercialization of EPNs include their effectiveness to a wide host range of insect pests, long shelf life, ease of mass production, and safety to the environment and non-target organisms. Various companies produce EPNs for commercial market purposes (Flexner and Belnavis 1999). The three largest companies that produce EPNs are Becker Underwood (USA), E-nema (Germany), and Koppert Biological Systems (Netherlands) (Abate et al. 2017). The commonly commercialized and globally distributed EPN species include *Steinernema feltiae* Filipjev (Rhabditida: Steinernematidae), *Steinernema carpocapsae* Weiser (Rhabditida: Steinernematidae), *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) and *Heterorhabditis indica* Poinar, Karunakar and David (Rhabditida: Heterorhabditidae) (Abate et al. 2017). *Steinernema feltiae* has been used to manage insect pests in different habitats such as leaf miners, scale insects, and thrips (Williams and Walters 2000; Arthurs and Heinz 2006; Negrisoli et al. 2013).

Orchards and forest plantations significantly contribute to economic growth and can serve as tools for poverty alleviation. Orchard systems play a major role in meeting human demand for food supply by producing high-value crops such as fruits and nuts and providing employment opportunities (Poulton and Poole 2001; Theron 2012; Achoja and Obodaya 2019). Likewise,

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forest plantations contribute to poverty alleviation and food security, as well as to social, economic and environmental values (Payne et al. 2015). However, insect pests threaten the sustainability of orchard systems and forest plantations and their roles in the economy (Thrupp 2000; Shaw et al. 2021). To avoid using chemical insecticides to suppress populations of insect pests in orchards and forest plantations, biological control agents such as EPNs can be used as alternatives.

The review highlights relevant examples intending to encourage the implementation of EPNs in the management of insect pests in orchards and forest plantations. We provide a brief overview of the infection process of EPNs and explore methods for applying EPNs to control soil-dwelling pests in orchards and forest plantations. We discuss the formulation of EPNs for field application and factors that affect their survival. In addition, we discuss approaches to improve EPN survival and efficacy post-application.

The infection process of entomopathogenic nematodes

The third stage of the nematode growth cycle called the infective juvenile (IJ) (Fig. 1.1), is the only non-feeding, free-living stage that is adapted to the soil conditions and responsible for searching for suitable hosts (Adams and Nguyen 2002). The IJs use three host-finding strategies as well as host-emitted chemical cues to locate the insect hosts. After locating the insect host, IJs use the natural openings (mouth, anus, or spiracles) to penetrate the host body and colonize the hemocoel, where they release the symbiont bacteria (Poinar Jr 1990; Forst et al. 1997). *Heterorhabditis* spp. use a dorsal tooth to penetrate through the cuticle of the insect host and access the hemocoel (Bedding and Molyneux 1982). The bacteria grow and multiply rapidly, and produce toxins and antibiotics that kill the insect host, prevent secondary infection by other pathogens, and convert the host body tissues into nutritious substances from which the nematodes feed (Akhurst 1993). Bacterial toxins result in septicemia which causes the death

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of the insect host within 24 – 72 h (Smart Jr 1995; Adams and Nguyen 2002). In some cases, however, mortality may be observed five days post-infection (Dillman et al. 2012).

After the IJs have successfully penetrated the insect host, they soon begin to feed and moult to the fourth-stage juveniles, which eventually moult to adults (male and female) of the first generation (Fig. 1.2). The females will oviposit eggs after mating with males, which hatch into the first-stage juveniles. The first-stage juveniles successively moult to second, third, and fourth-stage juveniles. The fourth-stage juveniles will become adults of the second generation (Adams and Nguyen 2002). Two to three generations can be reached depending on the availability of the food resources derived from the insect cadaver (Adams and Nguyen 2002). When food resources are running low, the second-stage juveniles of the current generation moults into the third-stage IJs that then exit the cadaver and search for a new insect host. For *Steinernema*, before exiting the cadaver the IJs incorporate the cells of the symbiont bacteria into a specialized vesicle inside the intestines, but IJs of *Heterorhabditis* incorporate the bacterial cells in their guts (Adams and Nguyen 2002). The difference between the life cycles is that the first generation adults of *Steinernema* are males and females whereas the adults of *Heterorhabditis* are hermaphroditic (Smart Jr 1995).

The formulation of entomopathogenic nematodes

The formulation of EPNs is the preservation of nematodes in such a manner that they can be applied using practical methods (Heriberto et al. 2017). The formulation is an important factor for the successful application of EPNs. The formulation consists of three major components, namely active ingredients, carriers, and additives. The active ingredients are the EPNs and carriers include solids, liquids, gel, or insect cadavers. Additives include surfactants, adsorbents, absorbents, UV radiation protectants, humectants, antimicrobials, and emulsifiers (Grewal 2002).

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Entomopathogenic nematodes are formulated for three purposes, namely storage, transportation, and direct application (Heriberto et al. 2017). Moreover, formulations are designed to facilitate the handling of nematodes while maintaining high viability, infectivity, and survival (Grewal 2002). The standard formulation should ensure high and consistent quality, high efficacy, and convenient application of nematodes (Heriberto et al. 2017). Selecting the appropriate formulation will increase the success of pest control (Shapiro-Ilan et al. 2006).

In this section, we review the formulation of EPNs for direct application in the field. Information about the formulation of EPNs for storage and transportation can be found in Georgis (1990), Shapiro-Ilan et al. (2001), Grewal (2002), Grewal and Peters (2005), Wright et al. (2005), Kim et al. (2015), Heriberto et al. (2017), Kary et al. (2021), and Nxitywa and Malan (2021). Georgis (1990) briefly highlighted the importance of carriers in the formulation that can partially desiccate nematodes or immobilize them, which in turn reduces their metabolic activities and improve viability. Grewal (2002) and Nxitywa and Malan (2021) provided a comprehensive review of nematode formulation, including formulation for storage and transportation, formulation for direct application, as well as factors that affect the survival of nematodes in the formulation.

Use of gels

According to Kaya and Nelsen (1985) encapsulating nematodes with calcium alginate gel beads can facilitate field application. Hiltpold et al. (2012) encapsulated *H. bacteriophora* in the alginate shell and applied it in the field to control *Diabrotica virgifera virgifera* LeConte. This formulation provided better results than when *H. bacteriophora* was sprayed on the soil (Hiltpold et al. 2012). However, nematodes were leaking from the capsules at room temperature. This was corrected by changing the reaction temperature needed to form the

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capsules and adding more Ca^{2+} to the alginate capsules (Kim et al. 2015). Furthermore, Kagimu and Malan (2019) showed that sodium alginate beads are a suitable formulation for *Steinernema jeffreyense* Malan, Knoetze & Tiedt 2015, *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams 2004 and *H. bacteriophora*. The alginate beads were able to retain the IJs from leaking for four weeks at 25°C. Such formulations allow EPNs to be stored for an extended time and transported for long distances.

Vermiculite and peat

Nematodes can be formulated by mixing them with vermiculite or peat. In this case, vermiculite or peat serves as the carrier. Vermiculite or peat formulation accommodates a high density of IJs and offers extended shelf life, which is better than sponge formulation (Grewal 2002; Nxitywa and Malan 2021). However, the application of vermiculite-nematode or peat-nematode mixture is labour intensive because it needs to be broadcasted or applied as a mulch (Georgis 1990). Conventional sprayers can be used to apply vermiculite-nematode or peat-nematode mixture. For this purpose, the sprayer nozzle needs to be removed. In some cases, however, the vermiculite or peat particles may be bigger to pass through the nozzle head and this will clog the spray equipment (Nxitywa and Malan 2021).

Insect cadavers

After infecting the insect hosts, IJs go through one to three generations in a period of 1-4 weeks and exit the cadaver when food resources are depleted (Koppenhöfer et al. 2020). This duration is influenced by temperature and differs between EPN species, insect host and their sizes (Mahmoud 2016; Subramanian and Muthulakshmi 2016). During this period, insect cadavers can serve as temporary storage and a source for the slow release of nematodes in the field (Heriberto et al. 2017). As a result, insect cadavers infected with nematodes have been considered another way to formulate EPNs (Fig. 1.3). Raja et al. (2015) highlighted studies

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reporting that this method has increased nematode dissemination, efficacy, and survival. The application of insect cadavers (last instar *Galleria mellonella*; Lepidoptera, Pyralidae) infected with *H. bacteriophora* in pot and field trials resulted in larval mortality of 95% and 56.87% of *G. mellonella*, respectively (Raja et al. 2015). Laboratory trials of insect cadavers (*G. mellonella* larvae) infected with *S. feltiae* resulted in 78.9%, 87.5%, and 98.2% larval mortality of codling moth buried at a depth of 1 cm, 2 cm, and 3 cm, respectively (Lacey et al. 2010). Dillon et al. (2007) broadcasted insect cadavers infected with *Heterorhabditis downesi* Stock, GriYn & Burnell, *S. carpocapsae*, or *S. feltiae* at the same time, around pine stumps to control the developmental stages of large pine weevil, *Hylobius abietis* Linnaeus, and observed reduced adult emergence of 53 – 76%.

Shapiro-Ilan et al. (2003) demonstrated that nematode-infected insect cadavers are a better application method than aqueous suspension concerning efficacy. In contrast, Dillon et al. (2007) reported that the application of nematodes using nematode-infected insect cadavers was equally effective as the aqueous suspension in controlling *H. abietis* on pine stumps. Bender et al. (2014) reported failure of *H. indica* to control diaprepes root weevil, *Diaprepes abbreviatus* Linnaeus, in the 5-year-old lemon grove when applied with nematode-infected cadavers of *G. mellonella*. The poor performance of *H. indica* may have been due to the clay soil, which influenced the movement and efficacy of EPNs.

Handling insect cadavers can however be challenging because they break easily and tend to stick together, this consequently affects the efficacy of nematodes (Shapiro et al. 2001). This was addressed by using kaolin starch mixture and unflavoured gelatin as coating materials to protect and conserve insect cadavers (Ansari et al. 2009; Del Valle et al. 2009). Insect cadavers can also be coated with clay to prevent them from breaking or sticking to each other (Grewal 2002). Machines designed specifically for improving the handling of insect cadavers are available. For example, Shapiro-Ilan et al. (2010) developed a machine that is used to package

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a large number of insect cadavers by wrapping them individually with tape. In addition, Spence et al. (2011) developed a technology that is used to desiccate cadavers of *G. mellonella* to improve handling and transportation.

Application methods

Application methods enable nematodes to access and infect the targeted insect pest (Georgis 1990). Selecting the appropriate application method will increase the chances of successful pest control (Georgis 1990; Shapiro-Ilan et al. 2006). When selecting the application method, it is important to consider the crop type, targeted pests, and area of application (Georgis 1990). Initially, the standard application method of EPNs was drenching, whereby the EPN aqueous suspension is directly poured into the soil. For convenience, agronomic or horticultural spray equipment and irrigation systems are used to facilitate the application of EPNs (Shapiro-Ilan and Dolinski 2012). Typical examples of irrigation systems used to apply nematodes include central pivot irrigation, trickle irrigation system, and furrow irrigation, whereas the agrochemical spray equipment includes hand-held sprays, knapsack pressure sprayers, mist blowers, electrostatic sprayers, boom sprayers, and aircraft sprayers (Georgis 1990; Wright et al. 2005; Shapiro-Ilan et al. 2006).

Irrigation systems

Irrigation systems are suitable for applying EPNs in the orchards and nurseries as opposed to the forest plantations that are usually rainfed. To apply nematodes with irrigation systems, EPN aqueous suspension is mixed with water in the tank and then applied during irrigation. The advantage of using irrigation systems for applying nematodes is that it requires minimal labour because irrigation equipment can be installed permanently and operated automatically (Georgis 1990). If the selected irrigation systems deliver the nematodes effectively without reducing their survival and density, a successful application can be achieved (Wright et al. 2005).

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Entomopathogenic nematodes have successfully controlled insect pests when applied with different irrigation systems. De Altube et al. (2008) used drip irrigation, ditch, and the subsurface injector to apply *S. carpocapsae* for controlling *Capnodis tenebrionis* Linnaeus in the apricot orchard and found no significant difference between the three application methods. Drip irrigation caused mortality of 95% whereas ditch and subsurface injection caused mortality of 92%. Similarly, Morton and García-del-Pino (2008) used the ditch and subsurface injection method to apply *S. feltiae* (1 million IJs/tree) to control *C. tenebrionis* in the cherry orchard and observed no significant difference between the application methods. The population of *C. tenebrionis* was reduced significantly with mortality ranging from 88.3 to 97%. *Steinernema riobrave* Cabanillas, Raulston & Poinar at a concentration of 20 IJs/cm^2 caused 97% mortality of *Helicovepa zea* Boddie when applied with furrow irrigation (Cabanillas and Raulston 1996). *Heterorhabditis bacteriophora* (Otinem formulation) provided mortality of 83% of *D. abbreviatus* when applied with a microjet irrigation system at a rate of 1 million IJs/tree (Downing et al. 1991). In addition, *S. riobrave* (400 IJs/cm^2) provided mortality ranging from 85% to 100% of soil-dwelling stages of *Conotrachelus nenuphar* Herbst when applied by pouring the nematodes' aqueous suspension in plots of the apple orchard (Shapiro-Ilan et al. 2013).

When applying EPNs with irrigation systems, certain aspects should be considered. It is critical to ensure that the water temperature in the tank and hoses remain between 4℃ and 30℃. This is because nematodes can die if exposed to temperatures above 35℃ for more than 30 minutes (Wright et al. 2005). Black hosepipes can easily heat up and this will increase the water temperature. Burying them will help to alleviate this problem (Wright et al. 2005). While in the tank, nematodes tend to sediment, with the largest nematodes sedimenting faster over time and this will cause poor distribution during application. Sedimentation of nematodes in the tank can be reduced by frequent agitation and the addition of carboxymethylcellulose in the water

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will help slow down the sedimentation speed (Peters and Backes 2003). Sedimentation also occurs in irrigation hoses, but increasing the flow rate can prevent it. The flow rate can, however, be reduced if irrigation hoses have holes (Wright et al. 2005). In addition, nematodes can also exit the irrigation hose through these holes; hence, it will be best to use hoses with no holes.

Spray equipment

Different spray equipment can be used for the application of nematodes depending on the size of the field. For example, hand-held sprayers, hose-end sprayers, and back-pack sprayers are suitable for use in small plots and home gardens (Grewal 2002). Equipment such as a tractor sprayer system, electrostatic spray system, and boom sprayers are appropriate to use in large plots such as orchards (Grewal 2002; Shapiro-Ilan and Dolinski 2012).

Dlamini et al. (2019) used a handheld spray to apply *S. yirgalemense* at rates of 0, 10, 20, and 40 IJs/cm^2 with the application rate of 40 IJs/cm^2 providing the highest larval mortality of 77.5% of *Phlyctinus callosus* Schonherr. Application of *S. yirgalemense* at 30 IJs/cm^2 with a 2 l adjustable-pressure handheld sprayer resulted in 86% mortality of the last instar larvae of *Thaumatotibia leucotreta* Meyrick (Steyn et al. 2019). Malan and Moore (2016) used a watering can to apply *H. bacteriophora* in the citrus orchard at concentrations of 20 and 80 IJs/cm^2 which resulted in 91% and 99% mortality of *T. leucotreta* larvae, respectively. Shapiro-Ilan et al. (2015a) used a boom sprayer, trunk sprayer, and watering can to apply *S. carpocapsae* for controlling peach tree borer, *Synanthedon exitiosa* Say. There was no significant difference between the application methods. Batista and Auad (2010) observed no significant difference between mortality obtained by *S. riobrave* and *Heterorhabditis amazonensis* Andaló, Nguyen, and Moino applied with a watering can and nematode-infected cadaver on the nymph of *Mahanarva spectabilis* Distant. Both application methods caused nymphal mortality of 71%.

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When using spray equipment for the application of nematodes, consideration should be given to spray components such as volume, agitation, pressure and recycling time, and spray distribution patterns. These components play a critical role in delivering nematodes (Grewal 2002). The holes of the screens and filters should be wide enough so that nematodes can pass through. It is also recommended to remove screens and filters (Grewal 2002).

Nematodes are sensitive to high pressure (more than 2070 kPa) and intensive recycling through the pumping system. High-pressure hydraulic pumps have high pressure which exerts a shear force on nematodes when passing through the nozzle filters (Wright et al. 2005). Roller pumps are recommended because they reach a pressure level that is not damaging to nematodes (Grewal 2002). EPNs differ in tolerance to different pressure levels. For example, *S. carpocapsae* and *H. bacteriophora* can withstand a pressure of up to 2000 kPa whereas *Heterorhabditis megidis* Poinar, Jackson and Klein can withstand a pressure of up to 1380 kPa (Wright et al. 2005).

Spray droplet size and spray distribution affect the amount of spray contents applied on the targeted surface. The fan and cone nozzles produce droplets that are big enough (more than 175 µm diam) to carry IJs of *S. carpocapsae*. Equipment used for irrigation and fertilizer application are suitable for the application of nematodes since they maximize the number of IJs carried per droplet (Wright et al. 2005). Furthermore, to improve the control of insect pests, nozzles with a high flow rate are recommended because they deposit a large amount of IJs on the targeted surface (Wright et al. 2005). Increasing pressure results in an increased flow rate and this in turn maximizes the number of IJs deposited per cm^2 (Lello et al. 1996).

Factors affecting the survival of EPNs

Various abiotic factors including ultraviolet (UV) radiation, temperature, soil moisture, and soil chemical and physical properties are critical for the survival of EPNs (Fig. 1.4). EPNs are

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sensitive to UV radiation and the effects of UV radiation differ between EPN species (Gaugler et al. 1992; Shapiro-Ilan et al. 2015b). Gaugler and Boush (1978) reported that UV radiation is lethal to nematodes. Brief exposure of nematodes to short UV radiation reduces their growth, pathogenicity, and reproduction potential. UV radiation can reduce the viability and virulence of EPNs, but the effects are more severe on virulence (Shapiro-Ilan et al. 2015b).

Temperature affects several aspects related to the nematode's biology, including growth, respiration, survival, dispersal, host-finding ability, and infectivity (Griffin 1993; Kaya and Gaugler 1993). The optimum temperature for survival of EPNs range from 4°C to 30°C (Grewal et al. 1994b). Li et al. (2021) reported that *Heterorhabditis beicherriana* LF strain Xing-Yue, Qi-Zhi, Nermut, Puza, and Mracek provided the highest mortality (>80%) of *Holotrichia parallela* Motschulsky at temperatures 21, 25, and 29°C, but provided the lowest mortality (<40%) at temperatures of 33 and 40°C. This suggests that the survival of *H. beicherriana* was high under the temperature levels of 21, 25, and 29, but declined as the temperature increased. Kung et al. (1991) observed that survival of *S. carpocapsae* is high at temperatures ranging from 5°C to 25°C, whereas survival of *Steinernema glaseri* Steiner is high at temperatures ranging from 15°C to 35°C. These results suggest that *S. carpocapsae* is cold-tolerant whereas *S. glaseri* is heat tolerant. Other EPN species that appeared to be heat tolerant are *Steinernema scapterisci* Nguyen and Smart, and *S. riobrave*, whereas *S. feltiae* is reported to be cold tolerant (Grewal et al. 1994b). Heat tolerant and cold tolerant EPN species are suitable for controlling insect pests that are active during summer and winter, respectively.

Nematodes require a film of water within the soil to move, hence their movement is restricted in desiccated soil (Kaya 1990). Moisture levels suitable for the survival of EPNs vary among EPN species. Excessive moisture leads to limited oxygen availability, whereas insufficient moisture restricts movement, which consequently reduces the survival of nematodes (Koppenhöfer et al. 1995). EPN species such as *S. carpocapsae* is reported to be better adapted

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to lower moisture levels than most EPN species (Koppenhöfer et al. 1995). At soil moisture of 13% and 18%, *H. beicherriana* provided larval mortality of over 80% of *H. parallela* (Li et al. 2021). The soil moisture of 18% is the optimum moisture condition for the survival of *H. beicherriana* since it provided the highest larval mortality (>90% 11 days post-inoculation) of *H. parallela* (Li et al. 2021).

Soil physical properties such as texture affect the movement and survival of nematodes. For example, soil with high clay content is characterised by small pore spaces and high compaction, which in return restricts the movement of nematodes and airflow (Kaya 1990). According to Kung et al. (1990), *S. carpocapsae* and *S. glaseri* have poor survival in clay soil but showed improved survival in sandy loam soil. The survival of nematode species differs depending on the soil type, but most EPNs survive better in soil with combined characteristics of sand and loam soil (Koppenhöfer and Fuzy 2006; Kanga et al. 2012).

Soil chemical properties such as pH can influence the survival of nematodes. EPN species are adapted to different pH levels, but the recommended optimum pH level for survival is between 4 and 8 (Kung et al. 1990). EPN species such as *Steinernema khoisanae* Nguyen, Malan, Gozel, *Steinernema tophus* Cimen, Lee, Hatting, Hazir, and Stock, *Steinernema beitlechemi* Cimen, Puza, Nermut, Hatting, Ramakuwela, and Faktorova, *Steinernema glaseri*, *Steinernema biddulphi* Cimen, Puza, Nermut, Hatting, Ramakuwela, and Faktorova, and *Steinernema Innovationi* Cimen, Lee, Hatting, and Stock had a survival of less than 50% when pH level exceeded 8. However, *S. carpocapsae* showed survival of more than 90% on soils with pH ranging from 3 to 11 (Khathwayo et al. 2021).

Several biotic factors related to the biology of nematodes are important for survival. This includes the host-finding strategy, virulence, tolerance to environmental conditions, and availability of insect hosts. The host-finding strategy of EPNs can indirectly affect the survival

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of EPNs by influencing the type of the targeted insect host. It is then necessary to match the host-finding strategy of EPNs to the specific insect pests (Shapiro-Ilan et al. 2006). The availability of suitable insect hosts is critical for the survival of EPNs (Mracek et al. 1999; Mráček et al. 2005). In the case where suitable insect hosts are abundant, EPNs with high virulence are likely to survive because they can infect, kill, and reproduce within the body of the insect host. Moreover, if EPNs are not tolerant to the prevailing environmental conditions, they are less likely to survive even if they are highly virulent and have the appropriate host-finding strategy for the specific insect host.

Soil is a habitat for many microbial and metazoan organisms some of which are natural enemies of EPNs (Kaya 2002). Within the soil environment, organisms such as bacteria, protozoa, mites, nematodes, and nematophagous fungi parasitize or prey upon EPNs (Kaya 2002; Raja et al. 2015; Stuart et al. 2015). Some of these organisms exist within the body of insects targeted by EPNs and hinder the growth of the nematode-bacterium complex (Skowronek et al. 2020). Other insect hosts possess body fluids that prevent the proliferation of the bacterial symbionts of the nematodes (Berkvens et al. 2014). The nematode-infected insect cadavers may be attacked by scavenging organisms which then reduces the density of EPNs (Kaya 2002). EPNs are subject to interspecific and intraspecific competition with other biological control agents for the same insect hosts. This results in reduced fitness or displacement by another species (Kaya 2002).

Persistence of entomopathogenic nematodes in the soil

As indicated in the previous section, the persistence of EPNs in the soil is affected by biotic and abiotic factors. Under favourable conditions, the persistence of EPNs depends on their potential to find and infect insect hosts and reproduce (Smits 1996). Five EPN species, namely *S. carpocapsae*, *S. feltiae* 4CFMO, *S. feltiae* EN02, *H. megidis*, and *H. downesi*, applied at a

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concentration of 3.5 million IJs/stump in 500 ml of water, persisted in the soil for up to three years (Dillon et al. 2008). The number of nematodes recovered from the soil remained high until the 36th month after which the number of nematodes declined. The long-term persistence of the five EPN species reported in that study was due to the presence of the larvae of *H. abietis*, which served as the recycling hosts for nematodes.

Heterorhabditis bacteriophora persisted for 23 months in a field used to grow beans and wheat. The long persistence was due to the larvae of *Sitona lineatus* Linnaeus detected from the field which served as recycling hosts. However, laboratory experiments demonstrated that *H. bacteriophora* persisted for 24.8 days in the absence of the host (Susurluk and Ehlers 2008). Wilson and Gaugler (2004) observed a poor persistence of *H. bacteriophora*, especially in the soil where mites and collembola were abundant.

Steinernema riobrave was more virulent to larvae of *D. abbreviatus* in Marl soil (80% silt and 15% clay) 25 days after inoculation than in flatwoods and ridge soils (both consisting of 90% sand). Its virulence was not significantly different in the latter two soil types (Shapiro et al. 2000). The persistence of nematodes was assessed for up to 25 days. Had the study continued, there was a chance that *S. riobrave* would have persisted for more than 25 days even though its persistence was declining over time. Higher numbers of *Steinernema scarabaei* Koppenhöfer and Fuzy IJs were recovered from sandy loam than in other soils (i.e. loamy sand, loam, silt loam, acidic sand, and potting mix). A high proportion of *S. scarabaei* IJs were recovered 28 days after inoculation in sandy loam whereas a small proportion was recovered 84 days after inoculation (Koppenhöfer and Fuzy 2006). *Heterorhabditis zealandica* Poinar was recovered from acidic soil 21 days after inoculation. The highlighted studies indicate that soil type and availability of susceptible insect hosts have a direct effect on the persistence of EPNs. This applies only if other abiotic and biotic factors are stable.

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Approaches to improve the survival and efficacy of EPNs after field application

Modification of the soil

Modification of orchard soils is an important practice to improve the survival and efficacy of EPNs. Treatment of soil with 20 to 30 tons/ha of animal manure increased the abundance of *S. feltiae* by three-fold whereas treatment with NPK fertilizers reduced the abundance of *S. feltiae* (Bednarek and Gaugler 1997). Duncan et al. (2007) also observed an increased abundance of nematodes from the soil in citrus orchards treated with animal manure. It was also observed that the application of animal manure decreased the prevalence of nematophagous fungi and increased the abundance of EPNs. Duncan et al. (2013) modified the citrus orchard that naturally had flatwoods soil with poor drainage by filling planting holes with sandy soil. As a result, a high diversity of EPNs and control efficacy on larvae of *D. abbreviatus* was observed on sandy soil more than on flatwoods soil. Due to the poor activity of nematodes on flatwoods soil, there was an increase in the population of *D. abbreviatus*, which in turn killed most trees in the orchard. Not only did the modification of orchard soil improve the abundance and efficacy of EPNs and decrease adult emergence of *D. abbreviatus*, but it also improved the survival, growth, and productivity of the trees (Duncan et al. 2013).

Maintaining optimum soil moisture

Pre- and post-application irrigation helps to improve the survival of EPNs (Grewal 2002). Pre-application irrigation is necessary to facilitate the movement of the nematodes, whereas post-application irrigation helps to wash the nematodes into the soil (Grewal 2002). Laboratory studies have shown that increasing moisture content resulted in improved nematode efficacy (Grant and Villani 2003; Ebssa et al. 2004). In addition, the virulence of nematodes lost due to soil desiccation can be restored by irrigating the soil (Grant and Villani 2003). *Steinernema riobrave* applied at a conentration of 20 IJs/cm^2 provided 96% and 97% mortality of corn

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earworm when applied post-irrigation and during irrigation, respectively (Cabanillas and Raulston 1996).

Timing of application

Successful pest control by EPNs depends on their interaction with the targeted insect pest. The interaction of EPNs and insect pests can be improved by matching nematode application with the occurrence of the susceptible developmental stage of the targeted insect pest (Georgis 1990). The effects of abiotic factors can also be alleviated by applying EPNs at specific times. For example, the negative impacts of UV radiation and temperature can be avoided by applying nematodes early in the morning or later in the evening (Shapiro-Ilan et al. 2006; de Waal et al. 2018).

Genetic improvement

Desirable traits such as host-finding ability, virulence, and tolerance to environmental conditions can be improved through selective breeding. For example, Gaugler et al. (1989) improved the host-finding ability of *S. feltiae* by 21 – 27 folds through 13 rounds of selective breeding. Similarly, Bal et al. (2014) improved the dispersal of *S. carpocapsae* by 21 – 37 folds through 10 rounds of selective breeding. Anbesse et al. (2012) were successful in improving the desiccation and heat tolerance of *H. bacteriophora* through selective breeding. Another way to improve EPN survival is to induce adaptation through slow and monitored exposure to stressors such as heat and desiccation (Baiocchi et al. 2017). EPNs with increased tolerance to adverse environmental conditions can have enhanced survival during and after application and ultimately provide better pest control.

Use of adjuvants

The host range of EPNs was initially restricted to insect pests found in the soil and cryptic habitats where conditions are less detrimental to nematodes (Gaugler and Kaya 1990).

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However, with the development of formulations with adjuvants, the host range of EPNs now includes insect pests found above ground (Lacey et al. 2006; Shapiro-Ilan et al. 2006; Lacey and Georgis 2012). Adjuvants significantly improve the survival of EPNs post-application in the field. Adjuvants such as antidesiccant and UV radiation protectants can be used to protect the nematodes against the effects of moisture deficiency and UV radiation (Baur et al. 1997). The application of a sprayable gel (Barricate) around the base of peach trees enhanced the survival and efficacy of EPNs against *S. exitiosa* (Shapiro-Ilan et al. 2015a). *Steinernema carpocapsae* applied in aqueous suspension that was mixed with an antidesiccant resulted in larval mortality ranging from 85% to 95% relative to 22% of control (Glazer et al. 1992). In addition, *S. feltiae* significantly controlled larvae of *Cydia pomonella* Linnaeus when it was combined with either of two adjuvants, namely Siltwet and Stock absorb (Lacey et al. 2006).

Target the susceptible developmental stage of the insect host

The developmental stage of the targeted insect host can influence the efficacy of EPNs. For most insect pests, the larval stage is more susceptible to EPNs than other developmental stages (Malan and Manrakhan 2009; Ebssa and Koppenhöfer 2012; Heve et al. 2017). When targeting the larval stages of a specific insect pest, some EPN species may be effective against young larvae whereas others may be effective against older larvae (Ebssa and Koppenhöfer 2012; Acharya et al. 2020). In contrast, the pupal and adult stages appear to be less susceptible to infection by EPNs, but this depends on the EPN species (Ebssa and Koppenhöfer 2012; Heve et al. 2017; Morris et al. 2020). Although adult and pupal stages of most insect pests are less susceptible to EPNs infection, *S. yirgalemense* at a concentration of 20 IJs/cm^2 was able to control 93% of cocooned pupae and the emerging adults of *T. leucotreta* (Malan et al. 2011). *Steinernema diaprepesi* Nguyen and Duncan at a concentration of 1000 and 5000 IJs/pupa caused 100% mortality of pupae of *Gonipterus platensis* Marelli (Damascena et al. 2020). In addition, *S. carpocapsae*, *S. feltiae*, and *S. riobrave* showed acceptable virulence against pupae

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of *Rhagoletis pomonella* Walsh (Usman et al. 2020). Therefore, to achieve better control, EPNs can be applied when the susceptible developmental stage of the insect pests is prevalent (Shapiro-Ilan et al. 2002).

Application rate

The nematodes must be applied at an appropriate rate to achieve successful infection. The recommended application rate for a small area is 25 IJs/cm^2 (2.5 × 10^9 IJs/ha), but this will differ depending on the EPN species, targeted insect pests, and soil conditions (Shapiro-Ilan et al. 2002; Shapiro-Ilan et al. 2006). Even lower rates can be applied and still provide acceptable results (Shapiro-Ilan et al. 2006). For a large area, the recommended application rate is 2.471 × 10^9 IJs/ha (Miles et al. 2012). Lacey et al. (2006) stated that the application rate of 1 billion IJs/ha is effective for controlling the codling moth. In addition, a concentration of 3.5 × 10^6 IJs in 500 ml of water (≈ 7.5 × 10^9 IJs/ha) per stump is standardized for successful control of the pine weevil (Dillon et al. 2006; Georgis et al. 2006).

Host-finding strategy

Entomopathogenic nematodes use three host-finding strategies, namely cruiser, ambusher, and intermediate (Adams and Nguyen 2002). Nematodes with cruising behaviour locate the insect host by following chemical cues emitted by the insect host, as well as cues from the tree host of the insect pest (Lewis et al. 2006). Examples of cues used include CO_2 and volatiles emitted from the insect host's cuticle and feces (Lewis et al. 1992; Lewis et al. 1993; Grewal et al. 1994a). Examples of nematodes with cruising behaviour include *H. bacteriophora*, *H. megidis*, *Steinernema anomaly* Kozodi, and *S. glaseri* (Grewal et al. 1994a). Nematodes with ambushing behaviour travel less distance and often wait in one place so that they can attach themselves to the insect hosts passing next to them. They are usually found near the soil surface. Examples of nematodes with ambusher behaviour include *S. carpocapsae* and *S. scapterisci*. Nematodes

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with intermediate foraging strategy (e.g. *S. feltiae*) have characteristics of both cruiser and ambusher nematodes (Grewal et al. 1994a). Based on different behaviours resulting from different host-finding strategies, the cruiser foragers are more effective at finding sedentary insect hosts deeper in the soil than ambushers, whereas the ambusher nematodes are effective at finding mobile insect hosts usually on or near the soil surface (Lewis et al. 2006). Hence, it is important to match the host-finding strategy of EPNs with the targeted insect pest.

EPNs can provide varying control levels depending on the host-finding strategy they use. *Heterorhabditis downesi*, a cruiser forager, reduced the adult emergence of *H. abietis* by 87%, whereas *S. feltiae* EN02, an intermediate forager, reduced adult emergence of the same insect by 67% and 30% in 2001 and 2002, respectively. In both years, *S. carpocapsae* All strain (ambusher forager) failed to reduce the number of adult emergence (Dillon et al. 2006). However, there was no significant difference in the percentage of soil-dwelling developmental stages of *H. abietis* parasitized by *H. downesi*, *S. feltiae* EN02, and *S. carpocapsae* All strain in 2003.

Certain EPN species can switch between different foraging strategies depending on the substrate they are exposed to (Kruitbos et al. 2010). For example, *S. carpocapsae* provided higher larval mortality of *Tenebrio molitor* Linnaeus than *H. megidis* in peat, but in the sand, *H. megidis* outcompeted *S. carpocapsae* (Kruitbos et al. 2010). Based on this observation, the authors suggested that both *S. carpocapsae* and *H. megidis* are habitat specialists. Another study demonstrated that *H. downesi* (81.7%) reduced adult emergence of *H. abietis* more effectively than *S. carpocapsae* (49.5%) in peat soil, a substrate from which *S. carpocapsae* is expected to perform better (Williams et al. 2015). EPN species with appropriate host-finding ability are more effective since they can infect the insect hosts while conditions such as soil moisture are still conducive (Lacey et al. 2006).

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Conclusion

The interaction of various biotic and abiotic factors affects the success of EPNs to control insect pests. To overcome the effects of these factors and improve the efficacy of EPNs, there is a need to investigate or develop superior EPN species or strains of improved virulence and tolerance to environmental conditions. Native EPN species or strains should be considered as they are better adapted to the local environmental conditions than exotic EPN species. For example, Abate et al. (2018) isolated six EPN species from South African plantations and indigenous forests. Such EPNs can be tested against insect pests occurring in the plantations and indigenous forests from which the EPNs were isolated.

The proper delivery of EPNs to the targeted insect pests improves control. EPNs are applied using application equipment originally developed for the application of agrochemicals. Developing effective and efficient novel application methods tailored for EPN applications can improve the efficacy of EPNs. For example, modifying the current application methods to consider the nematode's biology could reduce the mortality of EPNs inflicted by application equipment during application.

Initially, it was difficult to apply EPNs to control insect pests aboveground. However, over the years, there has been advancement in developing formulation strategies to facilitate the application and enhance the survival of EPNs. The formulations used to facilitate the application of EPNs aboveground are not as extensive as the formulation for the application of EPNs below the ground. This warrants the need for developing formulations that facilitate the application of EPNs aboveground. One way to do this would be to develop adjuvants or traps that lure the targeted insect pests to the point of EPN application. When applying EPNs aboveground, formulations should improve the survival of EPNs for long enough to enable them to search and locate the targeted insect pest without losing viability.

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Figure 1.1. Infective juveniles of *Steinernema yirgalemense*

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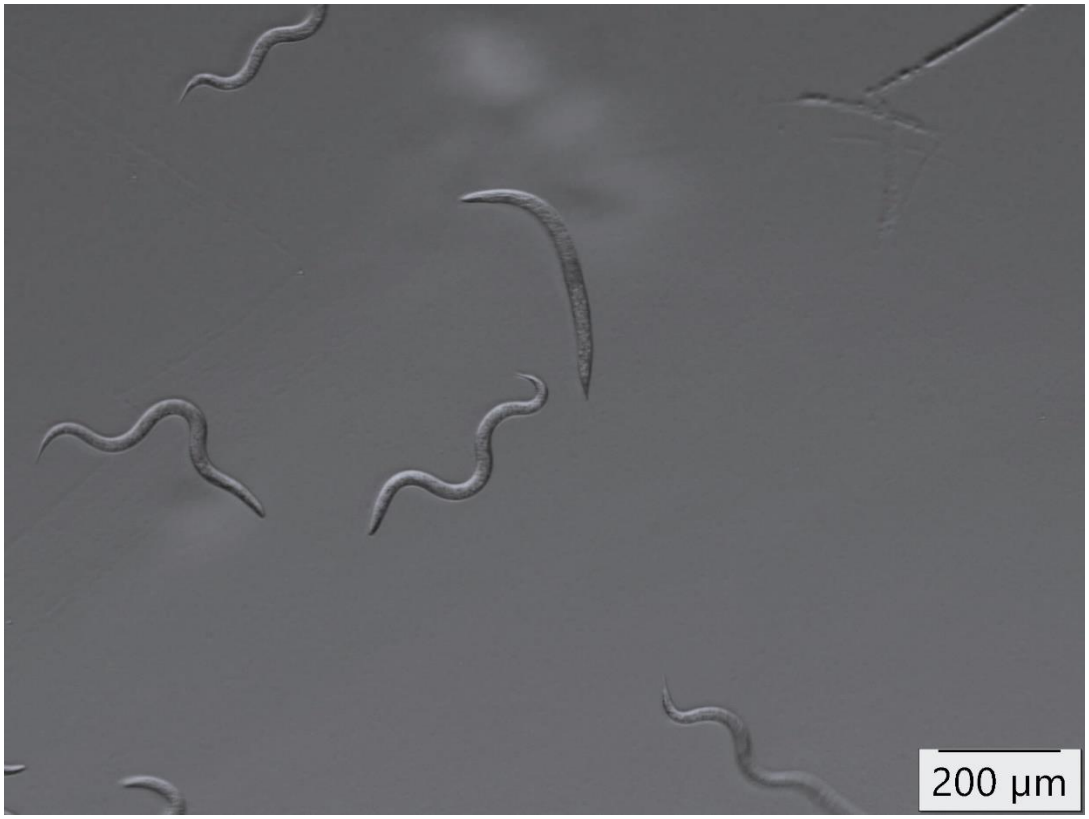

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Figure 1.2. The first generation male (A) and female (B) of *Steinernema bertusi*

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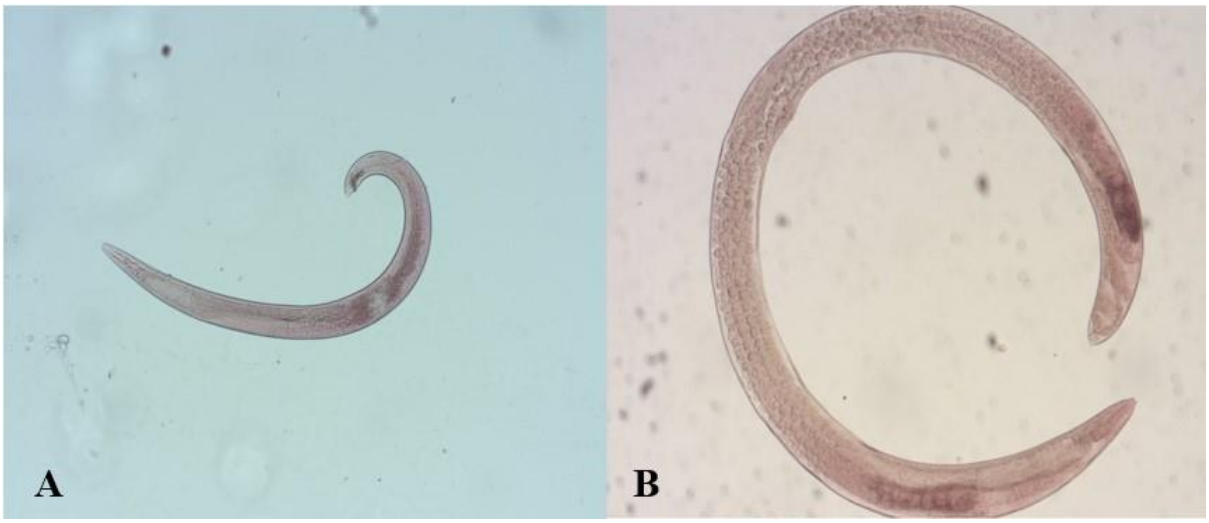

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Figure 1.3. Cadavers of *Galleria mellonella* infected with *Steinernema yirgalemense*

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Figure 1.4. The biotic and abiotic factors affecting the survival of entomopathogenic nematodes

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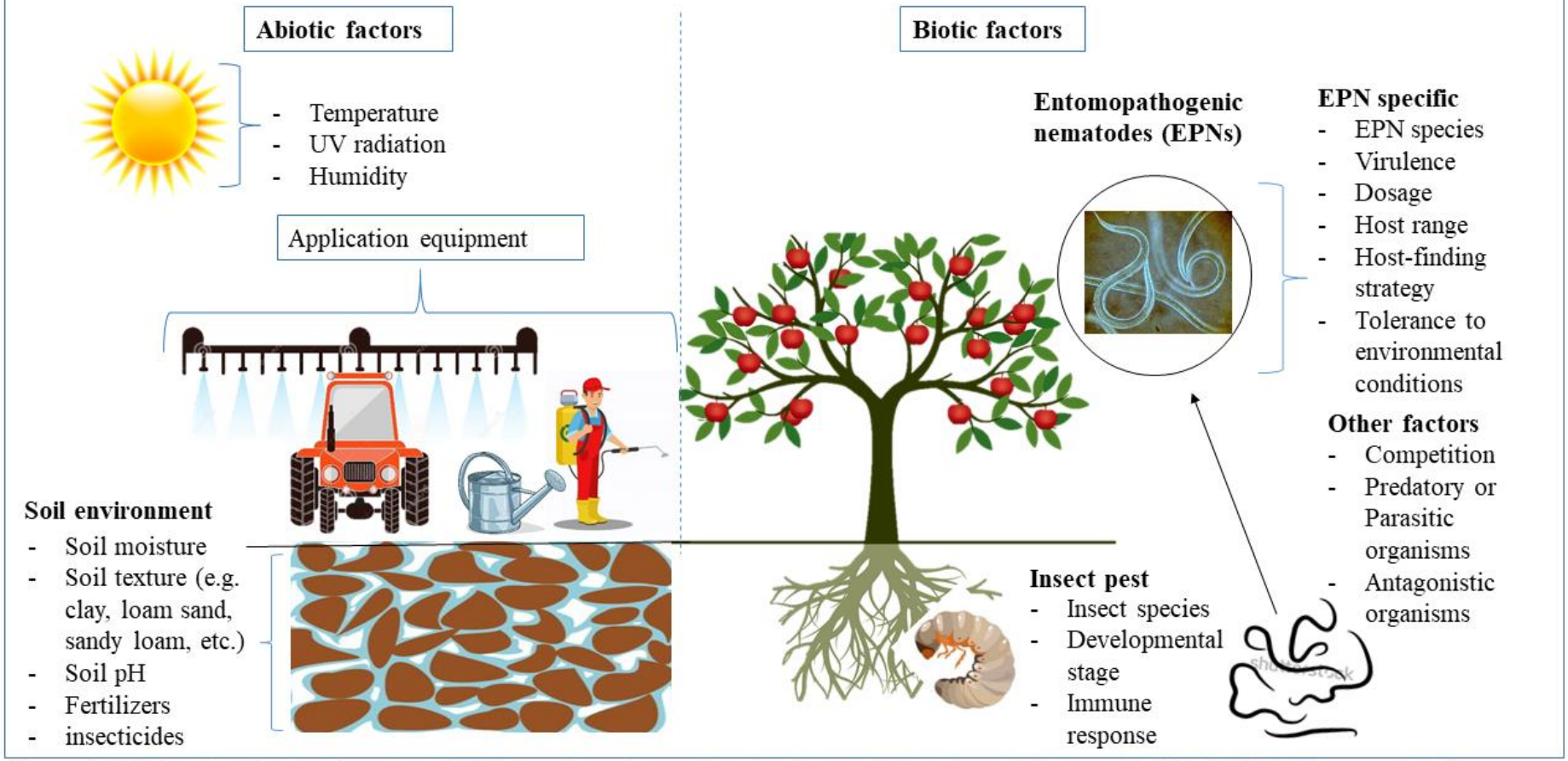
The biotic and abiotic factors affecting the survival of Entomopathogenic nematodes



Datta et al. 2017; https://www.baamboozle.com/game/106109, https://www.shutterstock.com/search/nematode, https://www.pinclipart.com/picdir/big/210-2107693_tomato-plants-clipart.png

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Chapter 2

Screening five local entomopathogenic nematode species for their virulence against pupae of *Gonipterus* sp. 2 under laboratory conditions

Abstract

Gonipterus sp. 2 is an important pest of eucalypt trees in South Africa and other countries. The pest is partially controlled with a mymarid egg parasitoid, *Anaphes nitens*. Identifying additional biological control agents that target other developmental stages of *Gonipterus* sp. 2 is necessary to improve control. Entomopathogenic nematodes (EPNs) are used as biological control agents for numerous soil pests and thus have the potential to be used against the pupal stage of *Gonipterus* sp. 2, which occurs in the soil. In this study, five South African local EPN species were screened for their virulence on pupae of *Gonipterus* sp. 2. At a concentration of 200 IJs/pupa in 50 µl of sterile water, *Steinernema jeffreyense*, *S. fabii*, *Heterorhabditis noenieputensis* and *H. safricana* provided pupal mortality of less than 40 %, while *S. yirgalemense* provided the highest pupal mortality of 100 % two days post-inoculation. *Steinernema yirgalemense* was selected and applied at different concentrations, namely 0, 12, 25, 50, 100, 200, and 400 IJs/pupa to determine its lethal concentration. Probit analysis indicated that the LC_{50} and LC_{90} of *S. yirgalemense* were 48.29 and 260.63 IJs/pupa, respectively. The study also showed that the pupal cases do not affect the efficacy of *S. yirgalemense* since it provided mortality of more than 90 % of pupae of *Gonipterus* sp. 2 still in their cases in soil bioassays. In addition, pupal age did not affect the efficacy of *S. yirgalemense*. Based on our findings, *S. yirgalemense* is a potential candidate for the biological control of pupae of *Gonipterus* sp. 2.

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Introduction

The increased invasion of non-native insect pests threatens the global eucalypt plantation forest industry (Hurley et al. 2016). The Eucalyptus snout beetle, *Gonipterus* spp. (Coleoptera; Curculionidae), native to Australia, is one of the invasive insect pests causing damage to *Eucalyptus* spp. grown in non-native areas. The beetle is now considered to be a species complex consisting of 10 cryptic species (Mapondera et al. 2012). One of these cryptic species, *Gonipterus* sp. 2, was detected in South Africa in 1916 and is now an established and important pest of *Eucalyptus* spp. in that country (Mapondera et al. 2012; Schröder et al. 2020). The adult and larval stages of this pest cause damage to the host plant by feeding on new leaves, shoots and buds (Mally 1924; Tooke 1955). In South Africa, the different growth stages of *Gonipterus* sp. 2, namely adult, egg, larval and pupal stages, overlap and occur throughout the year (Tooke 1955). The population peaks in September during spring when the host trees begin to produce new leaves (Tooke 1955).

Biological control with the egg parasitoid wasp, *Anaphes nitens* Girault, has been one of the main methods used to control populations of *Gonipterus* sp. 2 in South Africa and elsewhere in the world (Garnas et al. 2012; Schröder et al. 2020). *Anaphes nitens* was originally imported from Australia to South Africa in 1926 (Tooke 1955). However, the efficacy of *A. nitens* to suppress the population of *Gonipterus* spp. has not been consistent over the years (Reis et al. 2012; Schröder et al. 2020). For example, Loch (2008) reported that *A. nitens* was not as effective in cold highland areas in southwestern Australia and South Africa. In Portugal, *A. nitens* failed to control *Gonipterus platensis* Marelli in areas with altitudes higher than 400 m (Valente et al. 2017). Given the failure of *A. nitens* to provide consistent and widespread control of *Gonipterus* spp., exploring other biological control measures is necessary. Potential alternative biological control agents include entomopathogenic nematodes (EPNs), which are virulent to a wide range of insect pests (Kerry & Hominick 2002; Katumanyane et al. 2018).

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Entomopathogenic nematodes are soft-bodied non-segmented roundworms, naturally occurring obligate parasites of many soil-dwelling insects (Vashisth et al. 2013). They belong to the two families Steinernematidae and Heterorhabditidae. The steinernematid and heterorhabditid nematodes have a mutual relationship with bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively (Poinar Jr 1990). The free-living stage of these nematodes, called infective juveniles (IJs), is responsible for locating and infecting insect hosts. The IJs infect the insect host by penetrating through the insect's natural openings (mouth, anus, and spiracles) to gain access to the hemocoel, where they release their symbiont bacteria. The nematode-bacterium complex results in the death of the insect host within 24 h post-inoculation (PI) or after a few days depending on the EPN species and the insect host (Adams & Nguyen 2002; Adams et al. 2006; Dillman et al. 2012). The IJs grow and reproduce inside the insect cadaver and exit when they have depleted the food resources. Usually, they produce 2 - 3 generations before exiting the insect cadaver and searching for new hosts (Shapiro-Ilan & Dolinski 2012; Heriberto et al. 2017).

Entomopathogenic nematodes are environmentally friendly as they do not pose any known negative effects on non-target organisms and human health (Bathon 1996). They can be easily mass-produced using *in vivo* culture method for small-scale applications and *in vitro* (solid and liquid culture) methods for large-scale applications (Inman et al. 2012; Shapiro-Ilan & Dolinski 2012). EPN formulation techniques are used to facilitate long-term storage, transportation, and handling (Grewal 2002; Heriberto et al. 2017; Nxitywa & Malan 2021). In addition, EPNs can easily be applied using agrochemical spray equipment and irrigation systems (Georgis 1990; Wright et al. 2005; Shapiro-Ilan et al. 2006).

The larvae of *Gonipterus* sp. 2 spend most of their developmental time on the tree canopy feeding on leaves, where the application of EPNs is difficult as the EPNs will be exposed to detrimental factors such as ultraviolet radiation and desiccation (Begley 1990; Glazer et al.

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1992; Shapiro-Ilan et al. 2006). However, the fully-grown larvae of *Gonipterus* sp. 2 fall to the ground and form pupal cases with soil in which they pupate (Tooke 1955). While in the soil, the pupal stage lasts 15 - 17 days and adult beetles emerge after 12 – 15 days post-pupation (Tooke 1955). The duration that the pupae spend in the soil presents an ideal opportunity to apply EPNs.

To date, the virulence of EPNs on *Gonipterus* sp. 2 has not been investigated. The current study aimed to test the virulence of five local EPN species, namely *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler, and Adams*; Steinernema jeffreyense* Malan, Knoetze, and Tiedt; *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers, and Hurley; *Heterorhabditis noenieputensis* Malan, Knoetze, and Tiedt; and *Heterorhabditis safricana* Malan, Nguyen, De Waal, and Tiedt, on pupae of *Gonipterus* sp. 2. The tests were done under laboratory conditions by exposing uncased pupae of *Gonipterus* sp. 2 to the five EPN species. Uncased pupae of *Gonipterus* sp. 2 were exposed to the most effective EPN species to determine its lethal concentration in another experiment. The effect of pupal age of cased pupae of *Gonipterus* sp. 2 on the efficacy of the EPN species selected from the screening experiment was also determined.

Materials and methods

Source of insect and pupation soil

The larvae of *Gonipterus* sp. 2 were collected at Mondi's Mistley plantation (GPS coordinates: 29.218415 S and 30.679624 E), in KwaZulu-Natal province, South Africa. The soil for pupation was collected from the same plantation and was autoclaved before use for larval pupation. Larvae were placed in pillowcases with eucalypt leaves and transported to the insect rearing laboratory at the Biological Control and Insect Rearing facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, for further

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rearing until pupation. The larvae were reared in 1.2 l plastic containers lined with a paper towel containing eucalypt leaves as food for the larvae, which were replaced every second day. Fully-grown larvae were selected for different experiments.

Source of EPNs

EPN species used in the study were sourced from the FABI EPN collection. The EPN species used are listed in Table 2.1

Screening EPNs

Five local EPN species were screened for their virulence on uncased pupae of *Gonipterus* sp. 2. The field collected soil was autoclaved and allowed to cool overnight before use. The soil was then poured into 500 ml micro box containers and moistened with 10 ml of sterile water. Fully-grown larvae were transferred into the micro box containers (20 larvae per container), which were then incubated at 25 ℃ for 18 days to allow the larvae to pupate. After 18 days, pupae of *Gonipterus* sp. 2 were carefully removed from the pupal cases and used in the screening bioassays. The 12-well bioassay plates were used as the screening arena. Ten of the 12-wells were used in each plate. Each of the 10-wells, lined with 23 mm diameter filter paper, received one uncased pupa of *Gonipterus* sp. 2. Each EPN species (treatment) was inoculated on three bioassay plates (i.e. 3x10 pupae) at a concentration of 200 IJs per pupae in 50 µl of water using a pipette. Each bioassay plate was treated as a replicate. The three control plates received 50 µl of distilled water. The plates were covered with a damp paper towel and closed with their lids. The plates were then transferred into 2 l plastic boxes lined with a wet paper towel (100 % moisture). These plastic boxes were then closed with their lids and stored for 48 h in an incubator set at 25 ± 2 ℃.

After 48 h, pupae that showed color change and/or did not respond with a movement when poked with a soft paintbrush were considered dead. The dead pupae were removed from the

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bioassay plates, rinsed with sterile distilled water, and transferred into 9 cm diameter Petri dishes lined with filter paper to incubate for 48 h. Dead pupae from the same bioassay plate were transferred into one Petri dish. Mortality caused by the EPNs was further confirmed by dissecting the dead pupae 48 h PI and examining the presence of nematodes under a stereomicroscope. The experiment was repeated on a different date using a fresh batch of nematodes.

Probit test

The lethal concentrations (LC_{50} and LC_{90}) of *S. yirgalemense* against uncased pupae of *Gonipterus* sp. 2 was determined. Similar methods as explained for the screening bioassay were used. *Steinernema yirgalemense* was selected as it provided the highest pupal mortality during the screening bioassay. *Steinernema yirgalemense* was applied at different concentrations, namely 12, 25, 50, 100, 200, and 400 IJs/pupa. Each concentration was inoculated on three bioassay plates. Each plate, consisting of ten uncased pupae, was treated as a replicate.

The soil bioassay

The ability of *S. yirgalemense* to penetrate the pupal cases and infect the pupae of *Gonipterus* sp. 2 was tested in the soil bioassay. The soil was collected from Mondi's Mistley plantation in KwaZulu-Natal and teated as above. 90 ml vials each consisting of 40 g of soil moistened with 2 ml of sterile water were used as the infection arena. One larva was transferred into each vial. Larvae that had not burrowed into the soil after 24 h were replaced with new ones. The larvae were then incubated at 25 °C for 18 days to allow the larvae to pupate. After 18 days when the larvae had pupated, *S. yirgalemense* was administered per vial at concentrations of 12, 25, 50, 100, 200, and 400 IJs/pupa in 2 ml of sterile distilled water and was followed by an additional 1 ml of water to wash the IJs into the soil. The different concentrations were used to determine effective concentration for soil application. Each concentration was inoculated on

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ten vials and replicated three times to provide a total of thirty vials. On the 7th-day PI, the number of dead and live pupae and adult beetles (as some of the pupae had molted to adults) was recorded. Mortality caused by *S. yirgalemense* was confirmed by dissecting the dead individuals to check for the presence of nematodes with the aid of the stereomicroscope. Even though some larvae died due to the infection of *S. yirgalemense*, larval mortality was not recorded since the focus of this experiment was on pupal mortality. The mortality was determined by combining the number of dead pupae and adult beetles.

Pupal age experiment

The effect of pupal age on the efficacy of *S. yirgalemense* was tested. A similar method as explained in the soil bioassay experiment was used for the pupal age experiment. 90 ml vials were used as the infection arena, each consisting of 40 g of soil moistened with 3 ml of sterile water. One larva of *Gonipterus* sp. 2 was transferred into each vial and larvae that had not burrowed into the soil after 24 h were replaced with new ones. For this experiment, we inoculated *S. yirgalemense* on cased *Gonipterus* sp. 2 pupae of two age groups, namely 1-3 and 9-12 days-old. Inoculation of the early age (1-3 days old) and late age (9-12 days) pupae was done on the 18th and 25th days, respectively. The same batch of *S. yirgalemense* was administered for both age groups, at a concentration of 200 IJs/pupa in 2 ml of sterilized distilled water per vial. An additional 1 ml of water was added to wash the IJs into the soil. The control was prepared in the same way but received 2 ml of sterilized distilled water without nematodes. Each pupal age group consisted of ten vials that were replicated three times for a total of thirty vials. The inoculated vials were incubated in the dark at 25 °C for 7 days. On the 7th-day PI, the number of dead and live pupae as well as adult beetles were recorded. Mortality caused by *S. yirgalemense* was confirmed by dissecting the dead individuals to check for the presence of IJs under a stereomicroscope.

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Data analysis

The pupal mortality recorded in the screening experiment was subjected to two-way ANOVA (test date and EPN species as factors) for analysis using the RStudio statistical program (RStudio Team 2020). In the absence of a significant difference between the test dates and replicates of the same treatment, the data were pooled and analyzed with one-way ANOVA. One-way ANOVA was used to test the significant difference between mean pupal mortalities provided by EPN species. The mean pupal mortalities of EPN species were then separated with the Tukey post hoc test if there was a significant difference detected. Pupal mortalities for each EPN concentration were subjected to probit analysis in SPSS to determine the lethal concentrations of *S. yirgalemense* (IBM Corp 2021). The correlation between EPN concentration and pupal mortality was determined with RStudio. Data from the pupal age experiment was subjected to a t-test in RStudio to determine the effects of pupal age on the efficacy of *S. yirgalemense* (RStudio Team 2020).

Results

Screening EPNs

All five local EPN species were able to kill uncased pupae of *Gonipterus* sp. 2 (Fig. 2.1). Analysis using one-way ANOVA showed significant effects of the EPN treatments on pupal mortality ($f = 29.92$, $df = 5$, $p < 0.0001$). After 48h, pupal mortality of *Gonipterus* sp. 2 caused by *S. yirgalemense* (100 ± 0 %) was significantly higher than that caused by *H. noenieputensis* (36.67 ± 3.33 %, $p < 0.0002$), *S. jeffreyense* (23.33 ± 12.02 %, $p < 0.0001$), *H. safricana* (20 ± 10 %, $p < 0.0001$), *S. fabii* (10 ± 0 %, $p < 0.0001$), and the control (0 ± 0 %, $p < 0.0001$) (Tukey multiple comparisons of means; 95 % confidence level). Although *H. noenieputensis* provided the second-highest pupal mortality, it was not significantly different from the other three EPN

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species. The percentage mortality of the control did not differ from mortality obtained from *S. jeffreyense*, *H. safricana* and *S. fabii*.

Probit test

There was a significant effect of the concentration of *S. yirgalemense* on mortality of uncased pupae of *Gonipterus* sp. 2, 48 h PI ($f = 31.43$, $df = 5$, $p < 0.0001$, Table 2.2). Pupal mortality at 400 IJs/pupa did not differ significantly from mortality at 200 IJs/pupa but was significantly higher than pupal mortality at 100 IJs/pupa, 50 IJs/pupa, 25 IJs/pupa and 12 IJs/pupa. A positive correlation was observed between the EPN concentration and pupal mortality after 48 h PI ($r = 0.99$) (Fig. 2.2). According to the probit analysis, the regression line formula of *S. yirgalemense* is given by $y = 54.63x - 41.987$, where y is the mortality and x is the log 10 transformed concentration. The LC_{50} and LC_{90} of *S. yirgalemense* for *Gonipterus* sp. 2, calculated using the regression line formula, were 48.29 and 260.63 IJs/pupa, respectively.

The soil bioassay

There was a significant effect of the concentration of *S. yirgalemense* on cased pupal mortality of *Gonipterus* sp. 2, seven days PI ($f = 12.67$, $df = 6$, $P < 0.0001$, Table 2.2). On the 7th-day PI, the pupal mortality caused by the concentration of 400, 200, 100, 50, and 25 IJs/pupa was more than 90 % and there was no significant difference between the concentrations ($p = 0.84$). The concentration of 12 IJs/pupa caused the lowest pupal mortality of 41.67 % and was not significantly different from the control with 0 % mortality ($p = 0.16$). A moderate positive correlation ($r = 0.5$) was observed between the concentrations and mortality ($t = 2.4988$, $df = 19$, p-value = 0.0218).

Pupal age experiment

Pupal age did not affect the efficacy of *S. yirgalemense*, as mortality recorded from both age groups did not differ significantly ($t = 0$, $df = 4$, $p = 1$, Fig. 2.3). The mortality caused by *S.*

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yirgalemense on cased pupae of *Gonipterus* sp. 2 from the early and late age groups was 95.38 % and 100 %, respectively. Mortality of pupae for both age groups was significantly higher than in the control, i.e. natural mortality (P = 0.00014).

Discussion

The local EPN species used in this study (*S. yirgalemense*, *S. jeffreyense*, *S. fabii*, *H. noenieputensis*, and *H. safricana*) showed varied virulence on uncased pupae of *Gonipterus* sp. 2. Of the five EPN species tested, *S. yirgalemense* provided the highest mortality of uncased pupae and was selected for further experiments. The result of probit analysis showed that the LC_{50} and LC_{90} of *S. yirgalemense* are 48.29 and 260.63 IJs/pupa, respectively. Infective juveniles of *S. yirgalemense* at different concentrations, namely 12, 25, 50, 100, 200 and 400 IJs/pupa, penetrated the pupal cases of *Gonipterus* sp. 2 and provided mortality ranging from 41.67 % to 100 %. Pupal age did not affect the virulence of *S. yirgalemense*.

The current study showed that both cased and uncased pupae of *Gonipterus* sp. 2 are susceptible to local EPNs. This is in alignment with studies by Damascena et al. (2020) and Brida et al. (2021), which showed that pupae and pre-pupae of *G. platensis* (a species closely related to *Gonipterus* sp. 2) were susceptible to *Steinernema diaprepesi* Nguyen and Duncan and *Steinernema brazilense* Nguyen, Ginarte, Leite, dos Santos, and Harakava, respectively. In the current study, *S. yirgalemense* provided the highest mortality of uncased puape (100 ± 0 %) 48 h PI and it was significantly different from other EPN species. Other EPN species (*S. jeffreyense*, *S. fabii*, *H. noenieputensis*, and *H. safricana*) used in the study provided mortality of uncased pupae ranging from 10 % to 36.67 % and their effects on pupal mortality did not differ significantly. The mortality recorded included dead adult beetles because it is likely that pupae managed to molt into adult beetles post-infection, then succumbed to the infection as teneral adult beetles.

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In South Africa, several studies have reported varied mortality caused by *S. yirgalemense* and *H. noenieputensis* on different insect pests (Malan et al. 2011; James et al. 2018; Katumanyane et al. 2018; Platt et al. 2018; Dlamini et al. 2019). In the highlighted studies, both EPN species provided mortality ranging from 55 % to 94 %. *Heterorhabditis safricana* and *S. jeffreyense* at a concentration of 100 IJs/insect caused mortality of less than 5 % against female adults of *Planococcus ficus* Signoret and larvae of *Bradysia impatiens* Johannsen, respectively (Vieux and Malan 2013; Katumanyane et al. 2018). The current study also recorded mortality of less than 20 % from *H. safricana* and *S. jeffreyense* at a concentration of 200 IJs/pupa 48 h PI against *Gonipterus* sp. 2. Based on the results of the current study and the above-mentioned studies, we suggest that *Gonipterus* sp. 2 is not a suitable host for *S. jeffreyense*, *S. fabii*, *H. noenieputensis* and *H. safricana*, but a good host for *S. yirgalemense*, which is virulent to a wide range of other insect pests in South Africa.

The current study determined the LC_{50} (48.29 IJs/pupa) and LC_{90} (260.631 IJs/pupa) of *S. yirgalemense* against uncased pupae of *Gonipterus* sp. 2. Katumanyane et al. (2018) determined the LC_{50} (8 IJs/pupa) and LC_{90} (65 IJs/pupa) of *S. yirgalemense* against *B. impatiens*. The LC_{50} and LC_{90} of *S. yirgalemense* against larvae of *Lobesia vanillana* De Joannis were 7.335 IJs/larva and 62.761 IJs/larva, respectively (du Preez et al. 2021). Prinsloo et al. (2022) also determined the LC_{50} (4.38 IJs/larva) and LC_{90} (46.9 IJs/larva) of *S. yirgalemense* against the fifth instar larvae of *Thaumatotibia leucotreta* Meyrick. Similar methodology was used in the above-mentioned studies and the current study, thus the different LC_{50} and LC_{90} values are likely influenced by the host insect, demonstrating the variation in the virulence of the same EPN species across different insect pests and their developmental stages (Malan et al. 2011; Dlamini et al. 2020)

In most cases, the pupae of insect pests are less susceptible than other developmental stages to EPNs due to closed natural openings and barriers such as cocoons, which in turn reduces the

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infection success of EPNs (Lindegren et al. 1993; Abbas et al. 2001; Langford et al. 2014; Garriga et al. 2018). However, the current study has shown that, at concentrations of 25, 50, 100, 200, and 400 IJs/pupa, *S. yirgalemense* caused mortality of more than 90 % against pupae of *Gonipterus* sp. 2. The pupae were inoculated whilst in their pupal cases made of soil, and mortality was recorded on the 7th-day PI. Malan et al. (2011) also reported that *S. yirgalemense* at 800 IJs/ml concentration provided 93 % mortality of *T. leucotreta* pupae, 14 days PI in sand bioassays.

The current study showed that pupal age of *Gonipterus* sp. 2 does not affect the efficacy of EPNs. On the 7th-day PI at a concentration of 200 IJs/pupa, *S. yirgalemense* provided mortality of 95.83 % and 100 % of cased pupae of *Gonipterus* sp. 2 that were 1-3 and 8-12 days old, respectively. The mortality observed among the age groups did not differ significantly. This differs to what has been observed for the effect of EPNs on the larval stage of insects, where the larval instars can significantly affect the efficacy of EPNs (Shapiro et al. 1999; Ebssa and Koppenhöfer 2012; Acharya et al. 2020).

This is the first study in South Africa to report the potential of local EPNs to control pupae of *Gonipterus* sp. 2 with or without cases. Specifically, *S. yirgalemense* should be considered as a potential biological control agent for the pupal stage of *Gonipterus* sp. 2. In addition, more native EPN species should be tested for their virulence against *Gonipterus* sp. 2. Native EPN species not included in this study that could be tested are *Steinernema batswanae* Didiza, Lephoto, and Gray; *Steinernema beitlechemi* Cimen, Puza, Nermut, Hatting, Ramakuwela, Faktorova and Hazir; *Steinernema bertusi* Katumanyane, Malan, Tiedt, and Hurley; *Steinernema biddulphi* Cimen, Puza, Nermut, Hatting, Ramakuwela, and Hazir; *Steinernema citrae* Stokwe, Malan, Nguyen, Knoetze, and Tiedt; *Steinernema innovationi* Cimen, Lee, Hatting, Hazir, and Stock; *Steinernema khoisanae* Nguyen, Malan, Gozel; *Steinernema litchi* Steyn, Knoetze, Tiedt, and Malan; *Steinernema nguyeni* Malan, Knoetze, and Tiedt,

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Steinernema sacchari Nthenga, Knoetze, Berry, Tiedt, and Malan; and *Steinernema tophus* Cimen, Lee, Hatting, Hazir, and Stock. Promoting the use of native EPN species is important as it can help to avoid costs and regulations imposed on exotic EPN species (Abate et al. 2017).

Despite the promising results from this study on the potential of *S. yirgalemense* as a biological control agent for *Gonipterus* sp. 2, there are several challenges to the successful use of EPNs in the field for management of *Gonipterus* sp. 2. The pupal stage is cryptic within the soil (in small pupal cases made from soil particles) and this makes it difficult to determine the incidence of pupae to inform decisions on whether management, namely application of EPNs, would be required. For the same reason, post-application monitoring of the EPNs efficacy in the field, i.e. percentage pupal mortality, will also be difficult. The methods used to apply EPNs have different impacts on the efficacy, dispersal, and survival of EPNs (Shapiro-Ilan and Glazer 1996; Perez et al. 2003; Shapiro-Ilan et al. 2003). Therefore, research to investigate the timing of application and application methods that induce better dispersal of EPNs into the soil is necessary for successful field applications. Such methods include the use of insect cadavers infected with EPNs and an aqueous suspension of EPNs.

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Table 2.1. The local EPN species used in the study, their associated bacteria, place of origin and GenBank accession number.

EPN species	**Associated bacteria**	**Origin (province/town)**	**GenBank accession no.**	**Reference**
Steinernema yirgalemense	*Xenorhabdus indica*	Nelspruit, Mpumalanga	EU625295	(Malan et al., 2011)
S. jeffreyense	*X. khoisanae*	Jeffreys Bay, Eastern Cape	KC897093	(Malan et al., 2016)
S. fabii	*X. khoisanae*	Mpumalanga	KR527216	(Abate et al., 2016)
Heterorhabditis safricana	*Photorhabdus luminescens* subsp. *laumondii*	Western Cape	EF488006	(Malan et al., 2008)
H. noenieputensis	*P. luminescens* subsp. *noenieputensis*	Northern Cape	JN620538	(Malan et al., 2014)

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Table 2.2 Mean (± SE) pupal mortality of *Gonipterus* sp. 2 at different concentrations of *S. yirgalemense*. Different superscript letters on mortality values indicate significant differences.

Concentration (%)	Mortality[1] (%)	Mortality[2] (%)
400	100 ± 0^{a}	100 ± 0^{a}
200	76.67 ± 6.67^{ab}	95.24 ± 4.76^{a}
100	73.33 ± 3.33^{b}	95.24 ± 4.76^{a}
50	56.67 ± 6.67^{bc}	94.44 ± 5.56^{a}
25	33.33 ± 6.67^{cd}	90.48 ± 9.52^{a}
12	13.33 ± 6.67^{d}	41.67 ± 25.34^{b}
0 (control)	0^{d}	0^{b}

[1] Mortality of uncased pupae after 48 hours in 12-well bioassay plates
[2] Mortality of cased pupae after 7 days in soil

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Table 2.3. Mortality of uncased pupae *Gonipterus* sp. 2 caused by *S. yirgalemense* at different concentrations in 12-well bioassay plates

Concentration (IJs/pupa)	Replicates	Mortality			Survival			Mortality (%)
		Pupae	Adult	Total	Pupae	Adult	Total	
12	1	6	1	7	0	1	1	**87.5**
12	2	3	0	3	2	3	5	**37.5**
12	3	0	0	0	1	3	4	**0**
25	1	6	0	6	0	0	0	**100**
25	2	4	1	5	0	0	0	**100**
25	3	5	0	5	0	2	2	**71.43**
50	1	5	1	6	0	0	0	**100**
50	2	5	0	5	1	0	1	**83.33**
50	3	6	1	7	0	0	0	**100**
100	1	8	1	9	0	0	0	**100**
100	2	9	1	10	0	0	0	**100**
100	3	6	0	6	1	0	1	**85.71**
200	1	6	0	6	0	0	0	**100**
200	2	6	0	6	1	0	1	**85.71**
200	3	6	0	6	0	0	0	**100**
400	1	10	0	10	0	0	0	**100**
400	2	5	1	6	0	0	0	**100**

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400	3	8	0	8	0	0	0	**100**
Control	1	0	0	0	1	7	8	**0**
Control	2	0	0	0	2	4	6	**0**
Control	3	0	0	0	3	6	9	**0**

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Table 2.4. Mortality caused by *S. yirgalemense* on cased pupae of *Gonipterus* sp. 2 of two age groups in soil. Early age refers to pupae that are 1 – 3 day-old and late age refers to the pupae that are 7 – 12 days-old.

Age	Replicate	Mortality			survival			Mortality (%)
		Pupae	Adult	Total	Pupae	Adult	Total	
Early	1	7	0	7	0	0	0	**100**
Early	2	6	0	6	0	0	0	**100**
Early	3	7	0	7	0	1	1	**87,5**
Control	1	0	2	2	1	2	3	**40**
Control	2	0	1	1	2	5	7	**12,5**
Control	3	0	0	0	2	6	8	**0**
Late	1	1	8	9	0	0	0	**100**
Late	2	1	6	7	0	0	0	**100**
Late	3	0	9	9	0	0	0	**100**
Control	1	0	0	0	1	6	7	**0**
Control	2	0	0	0	1	7	8	**0**
Control	3	0	0	0	1	8	9	**0**

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Figure. 2. 1. The mean (±SE) mortality of uncased pupae of *Gonipterus* sp. 2 after 48 h exposure to five EPN species at a concentration of 200 IJs/pupa in 12-well bioassay plates. Different letters above the bars indicate significant differences ($p < 0.05$).

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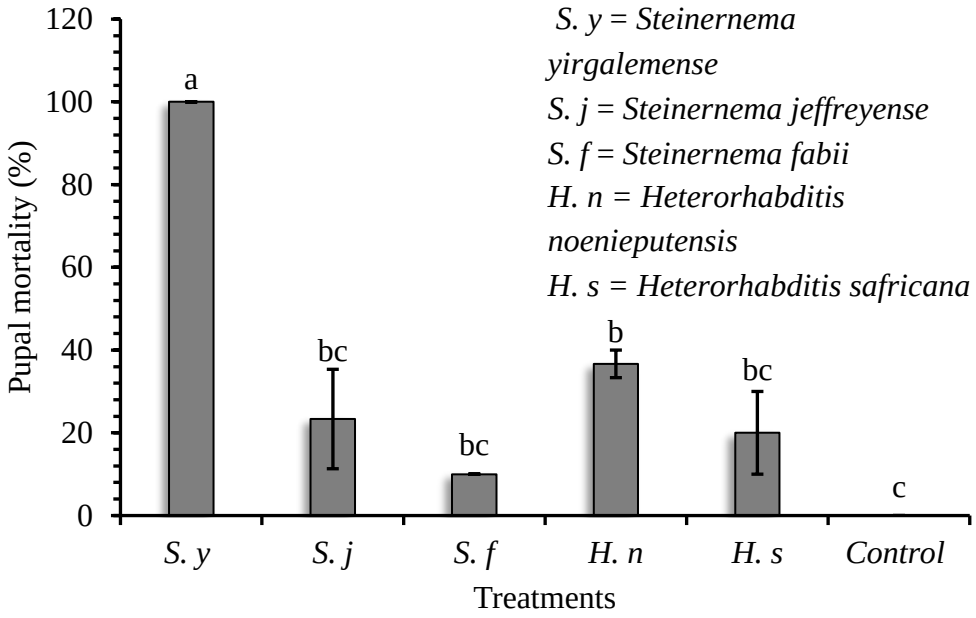

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Figure 2.2. The probit mortality of uncased pupae of *Gonipterus* sp. 2 by *S. yirgalemense* at different concentrations (Log): 12, 25, 50, 100, 200, 400 IJs/pupa, after 48 h PI in 12-well bioassay plates.

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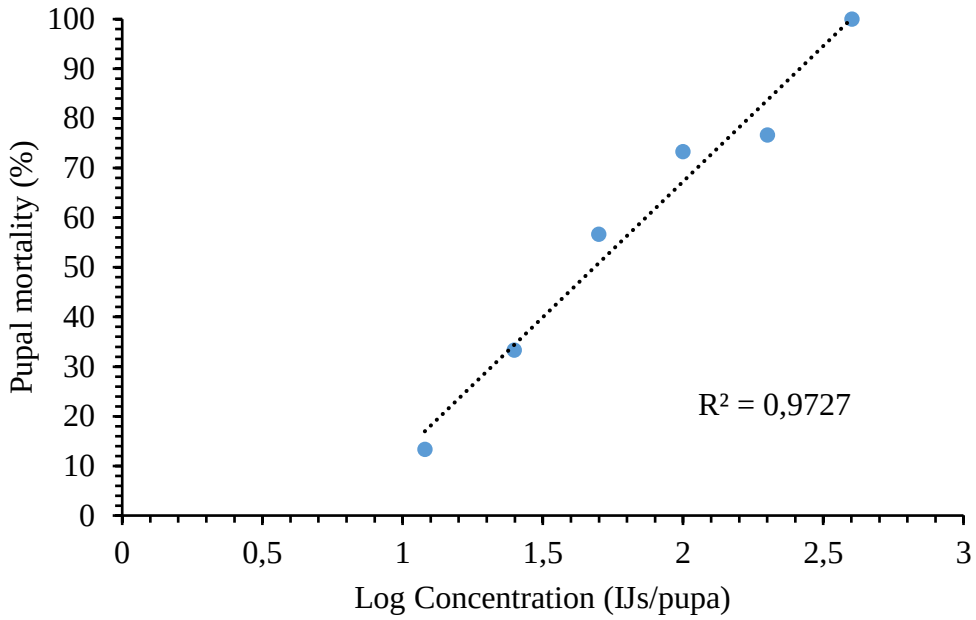

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Figure 2.3 Mean (±SE) mortality caused by *S. yirgalemense* on the 7th-day PI on uncased pupae of *Gonipterus* sp. 2 from two age groups, early and late age, in soil. Early and late pupal age refers to pupae that are 1-3 days old and 8-12 days old, respectively ($P < 0.0001$).

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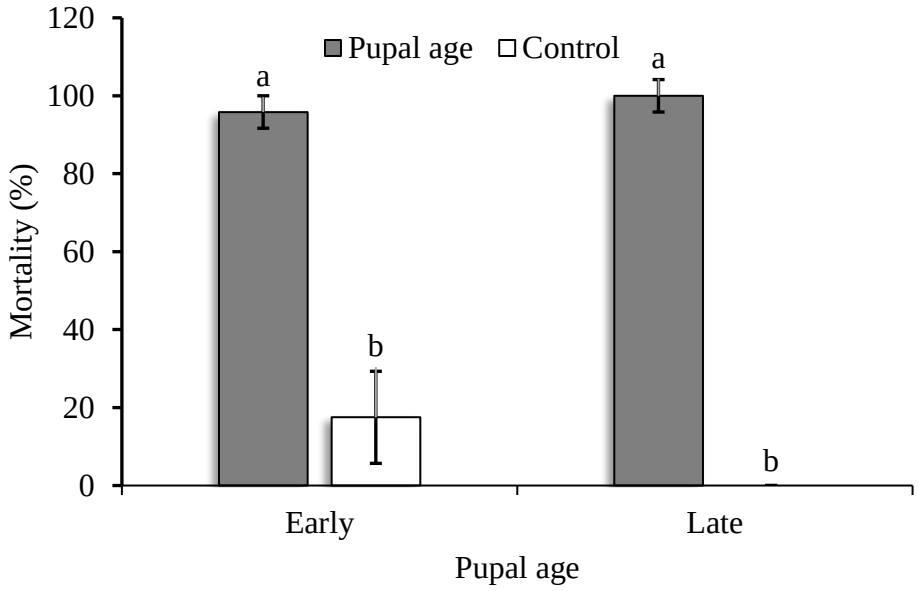

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Chapter 3

Host-foraging strategies of five local entomopathogenic nematode species in South Africa

Abstract

Entomopathogenic nematodes (EPNs) are obligate parasites of soil-dwelling insects and are used as biological control agents for many insect pests. These nematodes have a free-living third growth stage called infective juveniles (IJs), which are responsible for foraging and infecting suitable insect hosts. Infective juveniles exhibit three host-foraging strategies: cruising, ambushing, and intermediate foraging strategies. The foraging strategy of EPN is important for successful infection but is poorly understood. The current study investigated the host-foraging strategies of five local South African EPN species including *Heterorhabditis noenieputensis*, *H. safricana*, *Steinernema fabii*, *S. jeffreyense*, and *S. yirgalemense* by assessing their dispersal behaviour. Of the five EPN species, *H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense* showed a positive response to the presence of the wax moth larvae, whereas *S. fabii* showed a negative response. The four EPN species that showed a positive response to the presence of the host also provided 100% mortality of wax moth larvae that were buried in sand at a depth of 10cm, whereas *S. fabii* provided the lowest mortality of 34%. The average distance traveled by all five EPN species decreased on rough textured substrate compared with smooth textured substrate. The observed behavioural patterns suggested that *H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense* use a cruiser foraging strategy whereas *S. fabii* uses an ambusher foraging strategy.

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Introduction

Entomopathogenic nematodes (EPNs) are obligate parasites of many soil-dwelling insects (Jansson et al. 1990; Belien 2018). These nematodes belong to the families Steinernematidae and Heterorhabditidae, which have a symbiotic relationship with bacteria from the genus *Xenorhabdus* and *Photorhabdus*, respectively (Poinar 1990). The third growth stage of EPNs is called the infective juveniles (IJs) and is the only free-living stage of the nematodes. It is responsible for surviving the harsh environmental conditions in the soil (Smart 1995; Glazer 2002). These include various abiotic and biotic factors that have a negative impact on the efficacy of EPNs (Stuart et al. 2015). The main abiotic factors include ultraviolet radiation, temperature, soil moisture, and soil texture, whereas the biotic factors include an array of antagonist organisms, the interaction of EPNs with insect hosts, and the behaviour and ecology of EPNs (Kaya 2002; Stuart et al. 2015; Skowronek et al. 2020).

The IJs play an important role in searching for, locating and infecting suitable insect pests (Gaugler et al. 1997). Infective juveniles exhibit variation in behaviour when searching for insect hosts (Campbell et al. 2003). This difference in host-searching behaviour is categorized into two major host-foraging strategies: cruising and ambushing (Campbell et al. 2003; Lewis et al. 2006; Laznik and Trdan 2016). EPNs that use the cruising foraging strategy actively seek out the insect host by following the change in carbon dioxide gradient, volatiles emitted by the host and volatiles from plant roots induced by insect damage (Choo et al. 1989; Lewis et al. 1993; Adams and Nguyen 2002). EPNs that use an ambushing foraging strategy tend to use a sit and wait mechanism whereby they stand with their tails and wave part of their body to attack insect hosts passing within their striking range (Lewis et al. 2006). Unlike the cruising EPNs, the ambushing EPNs seldom use volatiles and do not disperse to a greater area (Grewal et al. 1993; Laznik and Trdan 2016). The third foraging strategy is the intermediate foraging strategy

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which includes EPNs that display characteristics of both cruiser and ambusher nematodes (Grewal et al. 1994).

A poor understanding of the EPN's behavioural ecology can result in inadequate pest control (Hominick and Reid 1990; Gaugler et al.1997). Each category of the host-foraging strategy influences the type of insect hosts that the EPNs infect successfully (Campbell et al. 2003). Matching the host-foraging strategy of EPNs with the target insect pest increases the efficacy of EPNs (Lewis et al. 1992). For instance, EPNs that use the cruising foraging strategy are effective against cryptic and sedentary insect hosts within the soil, while EPNs that use the ambushing foraging strategy are effective against mobile insect hosts found on or near the soil surface (Campbell et al. 2003).

The host-foraging strategies of EPN species are often inferred based on related species where the strategy has been studied (Lewis 2002). However, this is not an accurate approach and it is important to assess the host-foraging strategies of newly isolated EPN species to inform their use in pest management strategies and to broaden the existing knowledge of EPN host-foraging strategies. The aim of the current study was to investigate the host-foraging strategies of five EPN species that were isolated from South Africa (Malan et al. 2008; Malan et al. 2011; Malan et al. 2014; Abate et al. 2016; Malan et al. 2016). A few selected behavioural patterns that are linked to the host-foraging strategies were assessed. These were the responsiveness of the IJs to an insect host (wax moth larvae), the ability of IJs to detect and infect the insect host buried in sand at a depth of 10cm and the effect of different substrates (smooth versus rough) on the dispersal of the IJs. We predicted that EPNs that use cruiser foraging strategy will be attracted to the host volatiles and thus show greater movement towards the host insects; they will result in high larval mortality in sand bioassays, as they are able to move in the sand to locate the insect host; and that the difference in their movement on smooth and rough substrates will not be significantly different, because of they rarely nictate (standing with the tails and body wave).

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On the contrary, IJs that use an ambushing foraging strategy were predicted to not be attracted to host volatiles and thus show less movement towards the host insects; they will cause low larval mortality in sand bioassays; and the difference in their movement on smooth and rough substrates will be significant.

Materials and methods

Source of EPNs

The EPN species used in this study were locally isolated EPNs sourced from the EPN collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. These included *Heterorhabditis noenieputensis* Malan, Knoetze, and Tiedt; *Heterorhabditis safricana* Malan, Nguyen, De Waal, and Tiedt; *Steinernema fabii* Abate, Malan, Tiedt, Wingfield, Slippers, and Hurley; *Steinernema jeffreyense* Malan, Knoetze, and Tiedt; and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler, and Adams (Table 3.1). Standard procedures for the rearing and storage of EPNs were followed. In brief, 10 wax moth larvae, *Galleria mellonella* Linnaeus, were exposed to EPNs in a 9cm diameter Petri dish lined with filter paper for 24h (Kaya and Stock 1997). After 24h, the dead wax moth larvae were removed from the Petri dish to be rinsed with water and transferred into a new Petri dish lined with filter paper that was incubated for 48h. The dead larvae were then transferred into the White trap and the new batch of IJs that emerged and migrated into the water were harvested and stored in culture flasks at 12°C (White 1927). A new batch of IJs was used for the different replicates of experiments within three weeks after harvesting.

Source of insects

The wax moth larvae used in the study were sourced from the biocontrol and insect rearing facility at FABI, University of Pretoria. Wax moths larvae were reared following the adjusted method described by Birah et al. (2008). In brief, the newly hatched larvae were fed with a

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defined diet consisting of the following ingredients: powdered milk (200g), wheat bran (400g), oat bran (100g), nutty wheat bran (200g), yeast (100g), honey (300ml), and glycerol (400ml). The larvae were reared on this diet in jars kept in an incubator (Memmert IPS 750) until they reached the fourth instar. The fourth instar larvae were then collected for the experiments. The incubator was set at a temperature of 27 ± 1°C, relative humidity of 65 ± 5%, and 16:8 day: night photoperiod.

Responsiveness of IJs to the presence of a host

The responsiveness of IJs to the presence of wax moth larvae was assessed using 9cm diameter Petri dishes (Fig. 3.1). The methodology used by Grewal et al. (1994), Glazer and Lewis (2000) and Noosidum et al. (2010) was adjusted for this experiment. The lids of the Petri dishes were marked with two perpendicular lines to make four equal quadrants, followed by four concentric circles of 1, 2, 3 and 4cm in diameter from the center (Grewal et al. 1994). A 2% agar was prepared and 60ml of agar was poured into each Petri dish and allowed to cool for one hour (Glazer and Lewis 2000). Two holes of 3mm diameter were made on each lid, one hole at the edge of quadrant A and the other hole at the center (Fig. 3.1). The hole at the edge was used to accommodate a 1.5ml Eppendorf tube. Four holes were made at the bottom of the Eppendorf tube using a hot needle. The Eppendorf tube was fixed on the lid with a glue gun such that the bottom of the Eppendorf tube was suspended 3mm above the agar when the prepared lids were used to close the Petri dishes. The hole at the center of the lid of each Petri dish served as an entry port for the IJs. A small filter paper disc of 15mm diameter was placed at the center of each Petri dish on top of the agar to absorb water from the nematode suspension. The Petri dishes were closed with the prepared lids and sealed with parafilm.

The Eppendorf tube on each lid received one wax moth larva and this setup was kept at room temperature for one hour to allow larval volatiles to form. During this time the hole at the center

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was closed with transparent tape to avoid evaporation and drying out of agar. After one hour, 100 IJs were pipetted at the center, on top of the filter paper disc, in 50µl of water. IJs located in each section of quadrants A and C were counted every 10 minutes for a duration of 30 minutes after inoculation (Fig. 3.1). This was done with the aid of a stereomicroscope. The mean distance travelled by IJs toward the larva was calculated by the following formula (Noosidum et al. 2010):

$$\frac{\{(2 * A) + (3 * B) + (4 * C)\} - \{(2 * D) + (3 * E) + (4 * F)\}}{100}$$

The letters A, B, and C represent the number of IJs in the second, third, and fourth arc of quadrant A, respectively (Fig. 3.1). The letters D, E, and F represent the number of IJs in the second, third, and fourth arc of quadrant C, respectively (Fig. 3.1). The numbers in the equation, 2, 3 and 4 are distances in centimeters from the center (Glazer and Lewis 2000). One Petri dish was treated as a replicate and thus replicated five times for each EPN species. The control had a similar setup but without wax moth larva. The experiment was repeated twice on a different date.

Movement behaviour of IJs on a substrate with a smooth and rough texture

A similar setup as explained above was used here, except that the movement behaviour of IJs was investigated on a smooth and rough textured substrate in the absence of the host following the methodology by Noosidum et al. (2010). A cooled 2% agar contained in the Petri dish was regarded as a smooth substrate, whereas the rough textured substrate was prepared by evenly sprinkling 0.5g of sifted river sand on top of the cooled agar (Grewal et al. 1994). A small filter paper disc of 15mm diameter was placed (before sprinkling the sand particles for the rough substrate) at the center of each Petri dish on top of the agar to absorb water from the nematode suspension. The lids were secured on the Petri dish with parafilm. One hundred IJs in 50µl of distilled sterile water were pipetted on the filter paper in each Petri dish through the hole on

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the lid. The hole was then closed with transparent tape. The number of nematodes in each section of all four quadrants was counted every 10 minutes for a duration of 30 minutes with the aid of a stereomicroscope. The distance travelled by nematodes in any direction was calculated by the following formula (Noosidum et al. 2010):

$$\frac{\{(2 * A) + (3 * B) + (4 * C)\}}{100}$$

The letters A, B, and C represent the number of nematodes in the Petri dish's second, third, and fourth circles, respectively. The numbers in the equation 2, 3 and 4 are distances in centimeters from the center. Each EPN species/substrate combination was replicated five times and the experiment was repeated on a different date using a different batch of IJs.

Sand columns bioassay

Ten test tubes (2cm diam*eter*) were used as an arena. One wax moth larva was placed inside each test tube at the bottom and buried with sifted river sand at a depth of 10cm (Grewal et al. 1994). Stamping of the sand was minimized to keep compaction low, which allowed airflow and partial movement of the larva. A suspension of 100 IJs in 0.6ml of distilled sterile water was pipetted on top of the sand and the test tubes were closed with the lid and kept at 25℃. The number of dead larvae was recorded after 48h. The dead larvae were washed with a spray bottle to remove surface IJs and incubated at 25℃ for 24h in a 9cm diameter Petri dish lined with moist filter paper. The mortality by EPNs was confirmed by dissecting the dead larvae and checking for the presence of the IJs with the aid of the stereomicroscope. The ten test tubes were treated as a replicate and this was repeated five times per EPN species. The control setup received water without nematodes.

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Data analysis

The effect of two test dates on the movement of the nematodes was first checked with a t-test in R-studio (RStudio Team 2020). In the absence of a significant effect of the test dates on the movement of IJs, data were pooled and analyzed in one-way ANOVA to determine the difference in the responsiveness of EPN species to the presence of the host. Tukey post hoc test was used to separate the mean distance travelled by IJs toward the host post-detecting the difference in the responsiveness to the presence of the host. The student t-test was used to determine the difference in mean distance travelled by IJs between the smooth textured and rough textured substrate. One-way ANOVA was used to determine the difference between the mean larval mortality caused by EPN species in the sand column bioassays.

Results

Responsiveness of IJs to the presence of a host

There was a significant difference in the responsiveness of EPN species to the presence of wax moth larvae for all three-time intervals, namely 10 min ($F = 27.42$, $df = 4$, $p < 0.001$), 20 min ($F = 24.75$, $df = 4$, $p < 0.001$) and 30 min ($F = 24.23$, $df = 4$, $p < 0.001$). Four EPN species (*H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense*) had a positive net average distance (cm) per nematode towards the wax moth larvae at all three-time intervals, whereas *S. fabii* had a negative average distance away from the wax moth larvae (Table 3.2). *Steinernema jeffreyense* (1.21 ± 0.15cm, 1.15 ± 0.10cm, 1.13 ± 0.09cm) provided the highest average distance at 10 min, 20 min, and 30 min respectively, followed by *H. noenieputensis* (0.56 ± 0.11cm) at 10 min, and *S. yirgalemense* (0.67 ± 0.16cm and 0.73 ± 0.16cm) at 20 and 30 min, respectively. There was no significant difference in the average distance travelled by each EPN species (*S. jeffreyense*: $F = 0.12$, $df = 2$, $p = 0.89$; *S. yirgalemense*: $F = 0.72$, $df = 2$, $p = 0.50$; *H. noenieputensis*: $F = 0.18$, $df = 2$, $p = 0.84$; *H. safricana*: $F = 1.02$, $df = 2$, $p = 0.37$; and *S.*

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fabii: F = 0.32, df = 2, p = 0.73) towards or away from the wax moth larvae between the time intervals.

Movement behaviour of IJs on a substrate with a smooth and rough texture

There was a difference in the net average distance travelled by each EPN species between a smooth and rough textured substrate. *Steinernema yirgalemense* showed a significant decrease in the net average distance travelled on the rough substrate (0.49 ± 0.04cm, 0.54 ± 0.05cm, 0.54 ± 0.06cm) in comparison to the distance travelled on the smooth substrate (0.93 ± 0.16 cm, 1.12 ± 0.20 cm, 1.23 ± 0.22 cm) at 10 min (t = -2.55, df = 10.32, p = 0.03), 20 min (t = -2.76, df = 10.13, p = 0.02) and 30 min (t = -3.01, df = 10.31, p = 0.01), respectively. A similar trend was observed with *H. safricana* at 10 min (t = -5.13, df = 11.14, p < 0.001), 20 min (t = -6.19, df = 9.59, p < 0.001) and 30 min (t = -5.51, df = 9.59, p < 0.001). *Steinernema jeffreyense*, *S. fabii*, and *H. noenieputensis* also showed a similar trend, but in at least two time intervals (Table 3.3).

Sand columns bioassay

There was a significant difference in the mortality of wax moth larvae provided by the five EPN species 48h post-inoculation (pi) (F = 182.7, df = 4, p < 0.001). Four of these EPN species (*H. noenieputensis* (100% ± 0%), *H. safricana* (100% ± 0%), *S. jeffreyense* (100% ± 0%), and *S. yirgalemense* (99% ± 1%)) provided the highest mean (± SE) larval mortality and there was no significant difference between the EPN species (F = 1, df = 3, p = 0.40). *Steinernema fabii* (34% ± 4.76%) provided the lowest mean larval mortality. There was no larval mortality in the control.

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Discussion

This was the first study in South Africa to report on host-foraging strategies of local EPN species. The results suggested that four of the EPN species studied, namely *H. noenieputensis*, *H. safricana*, *S. jeffreyense* and *S. yirgalemense*, use a cruiser foraging strategy, whereas *S. fabii* possibly uses an ambusher foraging strategy. *Heterorhabditis noenieputensis*, *H. safricana*, *S. jeffreyense* and *S. yirgalemense* showed a positive average distance per nematode movement towards the wax moth larvae, in comparison to *S. fabii* which provided a negative average distance. The same EPN species that provided a positive average distance were also able to locate and infect wax moth larvae buried at a depth of 10cm and induce mortality of 100%. All five EPN species included in the study showed a decreased average distance travelled on the rough textured substrate compared to the average distance travelled on the smooth textured substrate.

A positive average distance towards the insect host by the four EPN species used in the study suggests that their IJs are attracted to the wax moth larvae. During the experiments, IJs of these EPN species were observed gathering underneath the Eppendorf tubes carrying the wax moth larvae, suggesting an intentional movement toward the host. This is similar to the results reported by Lewis et al. (1993) who demonstrated that *Steinernema glaseri* Steiner, a species that uses cruiser foraging strategy, locates its host by tracking associated cues. Grewal et al. (1994) also reported similar results on a few other EPN species, including *Heterorhabditis bacteriophora* Poinar; *Heterorhabditis megidis* Poinar, Jackson, and Klein; and *Steinernema anomaly* Kozodoi. *Steinernema carpocapsae* Weiser and *Steinernema scapterisci* Nguyen and Smart, EPNs that use an ambusher foraging strategy, seldom respond to the volatiles of an insect host and they travel less distance on a rough textured substrate (Grewal et al. 1994; Grewal et al. 1997). Similar results were observed in the current study where *S. fabii* rarely

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responded to the presence of wax moth larvae and travelled less distance on a rough textured substrate compared to the smooth textured substrate.

The ability of *H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense* to cause 100% mortality of wax moth larvae buried at a depth of 10cm suggested that these EPN species actively seek out their host. This was expected as the four EPN species showed directional responsiveness to the presence of wax moth larvae. Grewal et al. (1994) reported the same behavioural pattern exhibited by *H. bacteriophora*, *H. megidis*, and *S. anomali*, which located and infected wax moth larvae buried at a depth of 10cm. Similarly, an undescribed *Steinernema* spp. (isolate K8) showed positive responsiveness to the presence of the wax moth larvae and infected larvae buried at a depth of 9cm (Noosidum et al. 2010). Additionally, *H. safricana* was often observed burrowing in the agar and reaching the bottom of the Petri dish, which may indicate that the nematode is adapted to deep soil depths. *Steinernema yirgalemense* was able to infect pupae of *Gonipterus* sp. 2 buried at 5cm depth (Chapter 2 of this dissertation). This further supports the cruising foraging behaviour of this nematode observed in the current study. *Steinernema fabii* did not show directional movement towards the host but caused 34% mortality of wax moth larvae in the sand column experiment.

All five EPN species used in the study showed a decreased net average distance travelled on the rough textured substrate compared to the average distance travelled on the smooth textured substrate. This indicates an ambusher foraging strategy, as the rough textured substrate allows the nematodes to nictate (body wave), a behaviour associated with ambusher nematodes (Gaugler and Campbell 1993). Thus, ambusher nematodes are expected to reduce the distance travelled on a rough substrate, due to time spent nictating, whereas cruiser nematodes are expected to continue moving (Gaugler and Campbell 1993). However, these results are contrary to those from the other behaviours assessed in the study which suggested that the four EPN species (*H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense*) use cruiser

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foraging strategy. It is possible for EPN species to exhibit traits that are intermediary between the cruiser and ambusher foraging strategies but are categorized into a particular foraging strategy depending on the importance of the observed traits. For example, *Steinernema ceratophorum* Jian, Reid, and Hunt showed a high jumping rate and short duration standing bouts, suggesting that it is an ambusher forager, but was categorized as a cruiser forager as it was effective against sedentary insect hosts (Campbell and Kaya 2002). The reduced average distance travelled on the rough textured substrate by *S. fabii*, the negative response to the presence of the hosts and the low larval morality in sand bioassays suggest that this nematode uses an ambusher foraging strategy.

Increasing our knowledge about the biology of EPNs, particularly the foraging behaviours of their IJs, leads to an improved understanding of the potential of EPNs in pest management (Lewis et al. 2006). The EPNs that showed cruiser foraging behaviour in this study, namely *H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense*, are likely to be more effective against sedentary insect hosts, usually found in deep soil depths, compared to *S. fabii* which showed ambush foraging behaviour. This would include the pupal stage for forest insect pests that pupate in the soil, such as the Eucalypt snout beetle, *Gonipterus* sp. 2, a major pest of *Eucalyptus* spp. (see Chapter 2 of this dissertation). *Steinernema fabii* will be less effective against insect hosts in deep soil depths but will likely be more effective against mobile insect hosts found on/near the soil surface. This could include larvae of several insect pests that crawl on the soil surface before pupating.

Results from this study indicate the host foraging strategies used by five local EPN species, but further research is needed to confirm these results and better understand the strategies. This includes investigating other characteristics such as nictation, jumping and efficacy against a mobile host (Gaugler and Campbell 1993; Lewis 2002). In addition, there were several limitations in the current study that could have influenced the results. For example, the 9cm

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diameter Petri dishes could have restricted the dispersal of the nematodes and thus for future studies arenas with a larger area should be used to assess dispersal potential. Y-tube apparatus would be ideal to test the responsiveness of the IJs to the presence of the host as it allows a comparison of the preference of IJs between the arm with an insect host and the arm without an insect host (Boff et al. 2001). Counting the injective juveniles on the rough substrate was challenging as some of the IJs hid underneath the sand particles, and thus developing a method that will enable an accurate count of the IJs on a rough substrate is necessary.

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Table 3.1. The local EPN species used in the study, their associated bacteria, place of origin, and GenBank accession number.

EPN species	Associated bacteria	Origin (province/town)	GenBank accession no.	Reference
Heterorhabditis noenieputensis	*Photorhabdus luminescens* subsp. *noenieputensis*	Northern Cape	JN620538	(Malan et al. 2014)
H. safricana	*P. luminescens* subsp. *Laumondii*	Western Cape	EF488006	(Malan et al. 2008)
Steinernema fabii	*X. khoisanae*	Mpumalanga	KR527216	(Abate et al. 2016)
S. jeffreyense	*X. khoisanae*	Jeffreys Bay, Eastern Cape	KC897093	(Malan et al. 2016)
S. yirgalemense	*Xenorhabdus indica*	Nelspruit, Mpumalanga	EU625295	(Malan et al. 2011)

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Table 3.2. Net average movement ($\bar{x}$ ± SE) per IJ of five EPN species towards or away from wax moth larvae over time. The different superscript letters indicate the significant difference between results within a column.

EPN species	**Net average distance (cm/IJ) travelled towards (+) or away (-) from the host**		
	10 min	20 min	30 min
Heterorhabditis noenieputensis	0.56 ± 0.11^{B}	0.55 ± 0.10^{B}	0.63 ± 0.11^{B}
H. safricana	0.04 ± 0.06^{CD}	0.09 ± 0.05^{C}	0.14 ± 0.04^{C}
Steinernema fabii	-0.38 ± 0.10^{D}	-0.25 ± 0.11^{C}	-0.31 ± 0.12^{D}
S. jeffreyense	1.21 ± 0.15^{A}	1.15 ± 0.10^{A}	1.13 ± 0.09^{A}
S. yirgalemense	0.48 ± 0.13^{BC}	0.67 ± 0.16^{B}	0.73 ± 0.16^{AB}

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Table 3.3 The net average distance ($\bar{x}$ ± SE) per IJ travelled by EPN species on smooth and rough substrates at 10 min, 20 min, and 30 min.

EPN species	Average distance (cm) travelled by IJs between a smooth and rough substrate		
	10 min	20 min	30 min
Heterorhabditis. noenieputensis	0.65 ± 0.10/0.36 ± 0.05*	0.88 ± 0.15/0.52 ± 0.07*	1.08 ± 0.22/0.60 ± 0.07ns
H. safricana	0.68 ± 0.08/0.22 ± 0.03*	0.78 ± 0.09/0.23 ± 0.02*	0.81 ± 0.10/0.25 ± 0.02*
Steinernema fabii	0.73 ± 0.06/0.49 ± 0.07*	0.68 ± 0.09/0.55 ± 0.08ns	0.80 ± 0.06/0.58 ± 0.05*
S. jeffereyense	0.42 ± 0.06/0.36 ± 0.03ns	0.60 ± 0.07/0.40 ± 0.05*	0.76 ± 0.10/0.41 ± 0.05*
S. yirgalemense	0.93 ± 0.16/0.49 ± 0.04*	1.12 ± 0.20/0.54 ± 0.05*	1.23 ± 0.22/0.54 ± 0.06*

* indicates the significant difference between the average distance on smooth substrate and rough substrate per EPN species at each time interval. ns = non-significant

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Figure 3.1. Petri dish marked with four quadrants and four concentric circles of 1, 2, 3 and 4 diameters in centimeters from the center. A 1.5 ml tube with one wax moth larvae is placed at the edge of quadrant A. Infective juveniles are transferred to the center on top of the filter paper.

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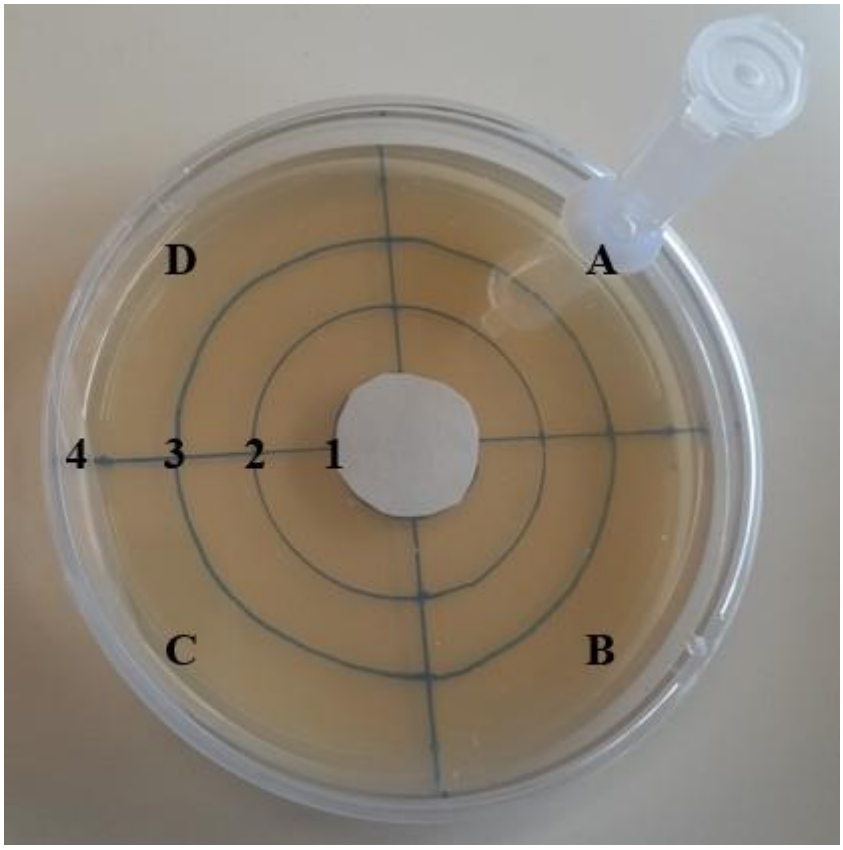

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Figure 3.2. Mortality ($\bar{x} \pm SE$) of wax moth larvae buried at a depth of 10 cm by five EPN species 48h PI. Different letters above the bars indicate significant difference in larval mortality.

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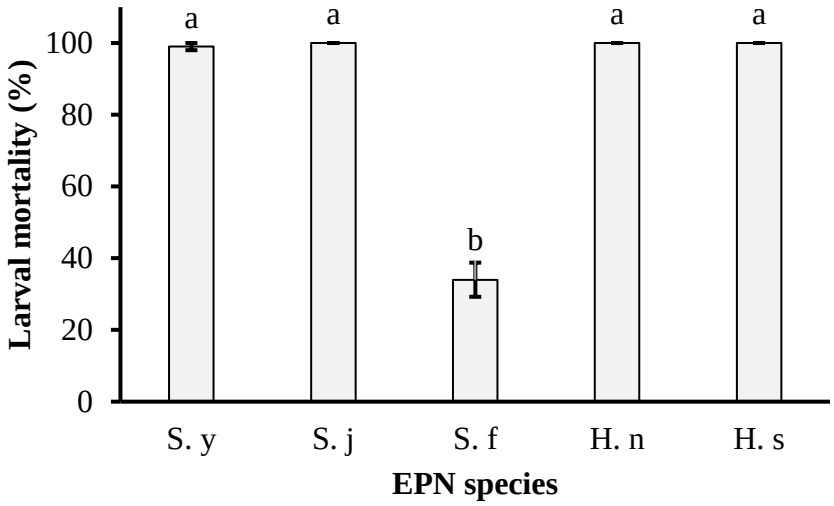

Key

H. n = Heterorhabditis noenieputensis

H. s = Heterorhabditis safricana

S. f = Steinernema fabii

S. y = Steinernema yirgalemense

S. j = Steinernema jeffreyense

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Chapter 4

Comparison of nematode-infected cadaver and aqueous suspension application methods on the efficacy of *Steinernema yirgalemense* against pupae of *Gonipterus* sp. 2

Abstract

During field applications, the infective juveniles (IJs) stage of entomopathogenic nematodes (EPNs) are generally applied in aqueous suspension using irrigation and spray equipment. Other methods for EPN application include using nematode-infected cadavers and carriers such as vermiculite, clay, and diatomaceous earth, which are mixed with EPNs to make formulations that can be applied in the field. Previous studies have shown that the choice of application method can affect the efficacy of EPNs. The current study compared the efficacy of *S. yirgalemense* against pupae of *Gonipterus* sp. 2 using two application methods, namely nematode-infected cadavers and an aqueous suspension, both applied in pot trials. *Steinernema yirgalemense* applied using both methods provided 100% mortality of pupae of *Gonipterus* sp. 2 with no significant difference between the application methods. These results suggest that the two application methods are potential candidate methods to apply IJs of EPNs to target pupae of *Gonipterus* sp. 2 in the field.

Introduction

The adoption of EPNs in pest management partly depends on their ease of application (Wright et al. 2005). For application purposes, EPNs are normally applied in aqueous suspension using spray equipment and irrigation systems, which are used for applying agrochemicals (Georgis 1990; Grewal 2002). The spray equipment used to apply EPNs ranges from simple tools such as knapsack sprayers to more sophisticated tools such as tractor-mounted sprayers and sprinkler

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irrigation systems, depending on the scale of application (Shapiro 2002). For example, knapsack sprayers and watering cans are ideal for applying EPNs on small plots such as home gardens, whereas tractor-mounted sprayers and sprinklers are appropriate for applying EPNs on large plots and orchards (Grewal 2002).

Other application methods include the use of carriers such as vermiculite, clay, or diatomaceous earth, which can be mixed with EPNs and directly applied in the field (Grewal 2002; Kagimu and Malan 2019). An additional method for direct application is the use of insect cadavers infected with EPNs (Shapiro et al. 2001). In most cases the insect cadavers are prepared by exposing wax moth larvae, *Galleria mellonella* Linnaeus, to EPNs (Shapiro et al. 2001). The dead wax moth larvae can be coated with clay, kaolin-starch combination, or masking tape to prevent them from breaking or sticking to each other during application (Shapiro et al. 2001; Grewal 2002; Ansari et al. 2009; Shapiro et al. 2010).

EPNs are prone to biotic and abiotic factors that hinder their survival, and some of these factors are unique to a particular application method (see Chapter 1 of this dissertation). The most important biotic factors include microbial and invertebrate antagonists and availability of suitable insect hosts. Abiotic factors include ultraviolet radiation, temperature, soil moisture, and soil chemical and physical properties (Stuart et al. 2015). Additionally, each application method has attributes that can affect the survival of EPN species during application. This includes detrimental conditions such as high pressure and shear force associated with sprayer application, which can indirectly affect the efficacy of EPNs (Fife et al. 2003; Fife et al. 2004).

The efficacy of application methods is not uniform across different EPN species and different environments. Some studies reported that EPNs applied in nematode-infected cadavers are better than those applied in aqueous suspension with spray equipment concerning infectivity and dispersal (Shapiro and Glazer 1996; Wu et al. 2018; Kaplan et al. 2020), whereas other

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studies reported that both application methods are equally effective (Brucks et al. 2005; Dillon et al. 2007; Batista and Auad 2010; Raja et al. 2015). It is clear that differences that may exist among application methods require extensive research to inform the appropriate application method for a particular EPN species in a particular environment.

In this chapter, the effects of two application methods on the efficacy of *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler, and Adams against the pupae of *Gonipterus* sp. 2 (Coleoptera: Curculionidae) were evaluated using pot trials. *Gonipterus* sp. 2 is a pest of eucalypt trees in South Africa and other parts of the world (Schröder et al. 2020). EPNs have been identified as a potential biological control option to target the soil-inhabiting pupal stage of this pest (see Chapter 2), but the method of application has not yet been investigated. *Steinernema yirgalemense* was chosen because it provided 100% mortality of pupae of *Gonipterus* sp. 2 in laboratory trials (Chapter 2). *Steinernema yirgalemense* was applied in nematode-infected cadavers of *G. mellonella* and aqueous suspension using a spray bottle.

Materials and methods

Collection and rearing of insects

The larvae of *Gonipterus* sp. 2 were collected from Mondi's Mistley plantation (-29.218415 S and 30.679624 E) in KwaZulu-Natal province, South Africa. *Gonipterus* sp. 2 larvae were placed in pillowcases with eucalypt leaves and transported to the insect rearing laboratory at the Biological Control and Insect Rearing facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, for further rearing. The larvae were reared in 1.2l plastic containers lined with a paper towel containing leaves of *Eucalyptus dunnii* Maiden as food for the larvae. The leaves of *E. dunnii* were replaced every

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second day. The larvae were reared until the pre-pupal stage before being used for experimental purposes.

Culturing nematodes

Steinernema yirgalemense was sourced from the FABI EPN collection. Infective juveniles (IJs) of *S. yirgalemense* were cultured in *G. mellonella* larvae as described by Kaya and Stock (1997) to obtain fresh batch of IJs. The dead larvae were transferred to the White trap to collect the IJs emerging from the cadaver (White 1927). The IJs were harvested, rinsed with water, and then stored in culture flasks at 12°C. The IJs were used within three weeks post-emergence.

Preparation of the nematode-infected cadaver

To obtain the *G. mellonella* cadavers, a Petri dish lined with a filter paper disc was inoculated with the aqueous suspension of 500 IJs of *S. yirgalemense* in 800µl of sterile distilled water, after which ten *G. mellonella* larvae were placed in the Petri dish. The lid of the Petri dish was secured with parafilm. The Petri dish was incubated for 48h after which the dead larvae were rinsed with sterilized distilled water and transferred to a new Petri dish that was incubated for eight days. The cadavers were used on the 10th day post-inoculation.

Preparation of the planting bags

Planting bags of 2l capacity were used as the assay arena. Each planting bag was filled with 0.5kg of sifted river sand, which was first autoclaved and cooled overnight. 50ml of sterile distilled water was added to the sifted river sand to bring the substrate to 10% soil moisture. Ten pre-pupae of *Gonipterus* sp. 2 were transferred into each bag, then kept in a greenhouse tunnel set at 25°C for 24h. Pre-pupae that had not burrowed into the soil after 24h were replaced with new ones. The planting bags were covered with a plastic bag to reduce moisture loss and kept for another 18 days to allow for pupation (Fig. 4.1).

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Evaluation of application methods

On the 18th day post introduction of *Gonipterus* sp. 2 pre-pupae, an additional 1.5kg of sifted river sand was added to each planting bag to give a pupation depth of 10cm. Infective juveniles of *S. yirgalemense* were transferred to the planting bags using two application methods that represented the treatments. In the first treatment, a 750ml capacity spray bottle was used to deliver an aqueous suspension consisting of 2500 IJs (1.49×10^9 IJs/ha) of *S. yirgalemense* in 150ml of sterile distilled water per planting bag (Fig. 4.2 B). In the second treatment, a quarter of a *G. mellonella* cadaver infected with *S. yirgalemense* was buried at a 2cm depth per planting bag (Fig. 4.2 A). The preliminary results showed that over 100 000 of IJs can be produced per cadaver of *G. mellonella*. Thus, a quarter of the larva was expected to produce ±25 000 (1.41×10^{10} IJs/ha) IJs. The sterile distilled water of 150ml was added per planting bag to achieve 10% soil moisture.

Each treatment was replicated 10 times with each planting bag consisting of ten pre-pupae of *Gonipterus* sp. 2 treated as a replicate. The planting bags were kept in the greenhouse tunnel set at 25°C for seven days. The same number of planting bags was used for the control, and each bag received 150ml of sterile distilled water without the nematodes. On the 7th day post-inoculation, the sand from each planting bag was sifted to collect and record dead pupae. The dead pupae were rinsed with water. If the IJs had not yet started to emerge from the pupae, the pupae were dissected to check for the presence of nematodes. The experiment was not repeated due to the unavailability of pre-pupae of *Gonipterus* sp. 2.

Data analysis

The means of pupal mortality from the two treatments, namely *S. yirgalemense* applied with nematode-infected cadavers and *S. yirgalemense* applied in an aqueous suspension, were compared with a t-test in Rstudio (RStudio Team 2020).

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Results

The results showed a significant difference between the application methods used to apply *S. yirgalemense* against pupae of *Gonipterus* sp. 2 relative to the control ($f = 486.3$, $df = 2$, $p < 0.001$). The nematode-infected cadavers and aqueous suspension used to apply IJs of *S. yirgalemense* provided pupal mortality of 100% and they were not significantly different from each other ($p = 1$, Fig. 4.1). Control mortality was less than 10%.

Discussion

Two methods of EPN application, namely nematode-infected cadavers and aqueous suspension, were tested for their effect on the efficacy of *S. yirgalemense* against pupae of *Gonipterus* sp. 2. The results showed no significant difference between the aqueous suspension and nematode-infected cadavers, as *S. yirgalemense* applied with these methods provided 100% mortality against pupae of *Gonipterus* sp. 2. Results from this study are in agreement with Parkman et al. (1993), wherein *Steinernema scapterisci* Nguyen and Smart applied in nematode-infected cadavers and an aqueous suspension with a watering can provided similar infection levels against the mole cricket, *Scapteriscus* spp. Raja et al. (2015) also found no significant difference between the four methods (i.e. nematode-infected cadaver, subsurface injection, spraying, and drip irrigation) used to apply *Heterorhabditis bacteriophora* Poinar against larvae of *G. mellonella*. Each application method provided a mortality of over 95%. The results of the current study further agree with several other studies such as Bruck et al. (2005), Dillon et al. (2007), and Batista and Auad (2010). However, other studies have obtained contradicting results. For example, Shapiro et al. (2003) reported that at seven days post-inoculation, *Heterorhabditis indica* Poinar, Karunakar, and David strain Hom1 reduced the survival of *Diaprepes abbreviatus* Linnaeus by 85% when applied with the nematodes-infected *Tenebrio molitor* Linnaeus cadavers compared to 42% when applied in an aqueous suspension.

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Similarly, *H. bacteriophora* showed improved infectivity against *G. mellonella* when applied in nematode-infected cadavers compared to when applied in an aqueous suspension (Shapiro and Lewis 1999).

Nematode application using infected cadavers have a number of traits important for a biological control agent (Griffin 1993; Griffin 2012). For example, nematodes applied with the nematode-infected cadavers are reported to have a better dispersal and tolerance to harsh environmental conditions than nematodes applied in an aqueous suspension (Shapiro and Glazer 1996; Gulzar et al. 2020). The apparent behavioural differences resulting in varied efficacy between nematodes in nematode-infected cadavers and aqueous suspension is due to the ascaroside pheromones. Ascaroside pheromones are associated with nematode-infected cadavers, and these pheromones can encourage the dispersal and infectivity of the nematodes emerging from the nematode-infected cadavers (Shapiro and Lewis 1999; Kaplan et al. 2020; Wang et al. 2022). However, our study and others have not found differences in the efficacy of nematodes applied in nematode-infected cadavers and nematodes applied in aqueous suspension. This could possibly be because the area on which the nematodes foraged and infected the host was small enough such that even lower concentration of nematodes would provide adequate efficacy irrespective of the application method used.

There were a number of limitations of this study that could have influenced the results. There were insufficient replicates due to the limited availability of pupae of *Gonipterus* sp. 2. The concentration of IJs in the nematode-infected cadaver method may have been too high for the area to which it was applied, and it was also higher than the concentration of IJs in an aqueous suspension. The recommended application rate is 25IJs/cm^2. Moreover, the greenhouse tunnel in which the experiments were conducted malfunctioned on various occasions causing the temperature to rise, thus potentially affecting the survival of the EPNs and pupae of *Gonipterus* sp. 2 and possibly increasing the percentage of natural mortality of *Gonipterus* sp. 2. Due to

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limitations on time and availability of the host insect, these flaws could not be addressed in this study, but future studies should consider these factors and make the required amendments.

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Figure 4.1. Planting bags containing sifted river sand and larvae of *Gonipterus* sp. 2. Leaves of *E. dunnii* were provided to the larvae as a food source.

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Figure 4.2. Application methods used to apply IJs of *S. yirgalemense*. A = cadavers of *G. mellonella* larvae infected with *S. yirgalemense*. B = spray bottle used to apply aqueous suspension of *S. yirgalemense.*

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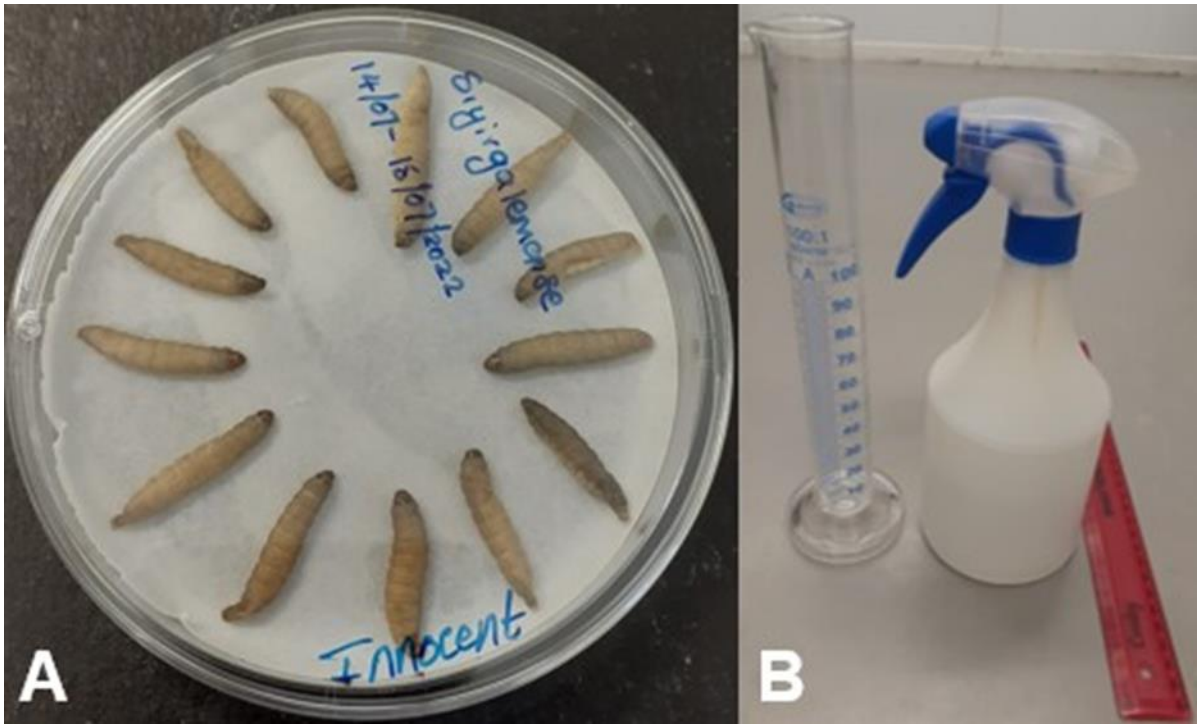

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Figure 4.3. Mean pupal mortality of *Gonipterus* sp. 2 by *S. yirgalemense* applied with nematode-infected cadavers and spray bottle. The letters above the bars indicate statistically significant differences between the application methods.

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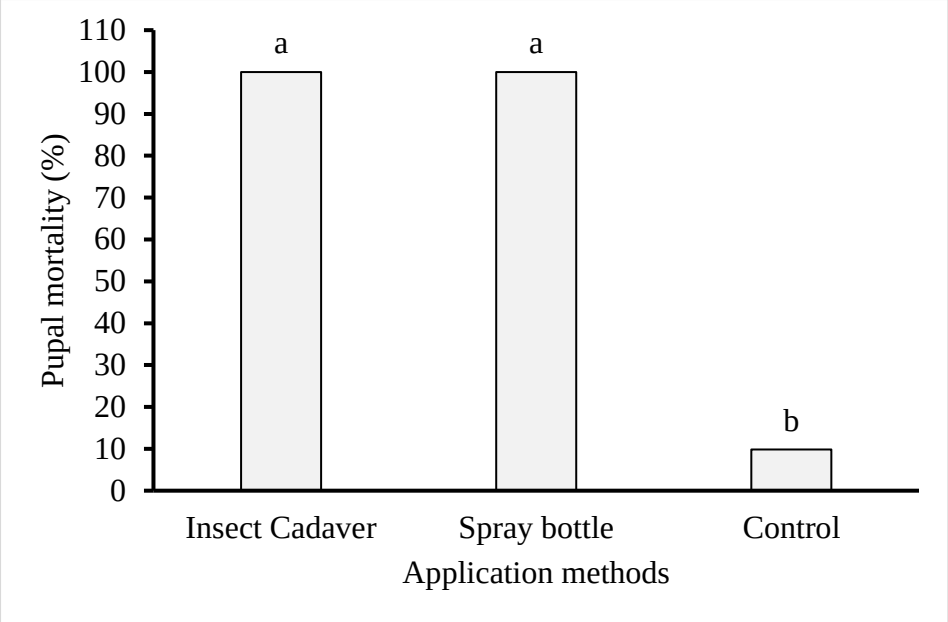

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Chapter 5

Concluding chapter

Several EPN species are commercialized on a global scale for use as biological control agents for many insect pests (Abate et al. 2017). However, there is still much to learn about the use of EPNs as biological control agents, especially the newly discovered species and isolates. In **Chapter 1**, I summarised the life cycle of EPNs and provided a comprehensive review of the formulation and application of EPNs. The factors that hinder the success of EPNs as biological control agents were reviewed and I suggested ways to remedy these factors. For example, measures that can be put in place to improve the success of EPNs includes genetic improvement of EPNs, use of adjuvants, modifying the soil environment, and selecting for genetically superior EPN species. The efficacy of EPNs can further be improved by targeting susceptible developmental stages of the insect pest, using effective application rates of EPNs, and matching the host-finding strategy of EPNs to the targeted insect pest. The use of EPNs to control insect pests in forest plantations is not extensive, thus Chapter 1 emphasized the need to incorporate EPNs into the management strategies of forest insect pests.

Gonipterus sp. 2 is an important pest of eucalypt plantations in South Africa (Mapondera et al. 2012). The adult beetles and larvae cause defoliation by feeding on the leaves of eucalypt trees thus reducing yield. An egg parasitoid, *Anaphes nitens*, was introduced as a biological control agent for *Gonipterus* sp. 2. However, alternative biocontrol agents such as entomopathogenic nematodes (EPNs) should be considered to target the pupal stage of *Gonipterus* sp. 2. **Chapter 2** reported on the virulence of five South African EPN species (*Heterorhabditis neonieputensis*, *H. safricana*, *Steinernema fabii*, *S. jeffreyense*, and *S. yirgalemense*) against uncased pupae of *Gonipterus* sp. 2 in 12-well bioassay plates. *Steinernema yirgalemense* caused 100% mortality of uncased pupae of *Gonipterus* sp. 2, whereas the remaining EPN species caused pupal

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mortality of less than 40%, 48h post-inoculation (PI) at a concentration of 200IJs/pupa. In alignment with this study, *S. yirgalemense* caused mortality ranging between 50% and 100% against a variety of insect pests in South Africa (Steyn et al. 2017; Katumanyane et al. 2018; Platt et al. 2018; Dlamini et al. 2019; Dlamini et al. 2020).

The lethal concentration of *S. yirgalemense* on uncased pupae of *Gonipterus* sp. 2 in 12-well bioassay plates was determined to further examine its efficacy. The LC_{50} was 48.29 IJs/pupa and the LC_{90} was 260.631 IJs/pupa. These values are substantially higher than the LD_{50} and LD_{90} of 8.98IJ/larva and 64.16IJ/larva, respectively, provided by *S. yirgalemense* against larvae of *Bradysia impatiens* (Katumanyane et al. 2018). This suggests that *S. yirgalemense* needs a higher concentration to kill the uncased pupa of *Gonipterus* sp. 2 than the larva of *B. impatiens*. The lethal concentration values of EPNs depend on the susceptibility of the insect pest and vary across different insect pests being targeted and their developmental stages (Katumanyane et al. 2018; du Preez et al. 2021; Prinsloo et al. 2022). Therefore, the lethal dosage can be used to inform the appropriate application rate of EPN species against a specific insect pest. *Steinernema yirgalemense* also provided mortality of more than 90% against cased pupae of *Gonipterus* sp. 2 at different concentration levels in soil bioassays. These results are different from those of other studies which reported that pupae of several insect pests are resistant to EPNs as they have closed natural openings and can create cocoons to serve as barriers that reduce infection by EPNs (Abbas et al. 2001; Langford et al. 2014; Garriga et al. 2018). The results observed in this study demonstrate the potential of *S. yirgalemense* to be applied in the field to target pupae of *Gonipterus* sp. 2.

The efficacy of EPNs can be improved by understanding host-foraging strategies of their Infective Juveniles (IJs). **Chapter 3** investigated the host-foraging strategies of IJs of five South African EPN species by studying aspects of their movement and behaviour. Four EPN species (*Heterorhabditis noenieputensis, H. safricana, Steinernema jeffreyense,* and *S.*

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yirgalemense) were attracted to the wax moth larvae, and resulted in high larval mortality in sand bioassays, suggesting that these EPNs use a cruiser foraging strategy. These results agree with Grewal et al. (1994) who reported similar behavioural patterns exhibited by *Heterorhabditis bacteriophora*, *H. megidis*, *Steinernema anomaly*, *S. carpocapsae*, and *S. scapterisci*. However, not all the results from our study confirmed that the four EPNs (*H. noenieputensis*, *H. safricana*, *S. jeffreyense*, and *S. yirgalemense*) followed a cruiser foraging strategy, and thus further work is needed. *Steinernema fabii* showed a negative response towards the wax moth larvae at the bottom of the sand columns and thus resulted in low mortality. *Steinernema fabii* further showed a reduced distance travelled on the rough textured substrate compared to the distance travelled on the smooth textured substrate. These behavioural patterns are associated with EPNs that use an ambushing foraging strategy (Grewal et al. 1993; Grewal et al. 1997).

Infective juveniles of EPNs can easily be applied with various spray and irrigation methods that are normally used for the application of agrochemicals. The choice of application method can impact the efficacy of EPNs. In **Chapter 4**, the effects of the nematode-infected cadaver and aqueous suspension application methods on the efficacy of *S. yirgalemense* against pupae of *Gonipterus* sp. 2 were tested. No differences were observed between the nematode-infected cadaver and aqueous suspension application methods on the efficacy of *S. yirgalemense* as both methods provided 100% mortality of pupae of *Gonipterus* sp. 2 that was buried in sand. This is in alignment with results from several other studies which reported that the application methods used had no effect on the efficacy of EPNs (Brucks et al. 2005, Dillon et al. 2007, Batista and Auad 2010, and Raja et al. 2015). However, our results are not consistent with Shapiro et al. (2003) who reported that *Heterorhabditis indica* applied in nematode-infected cadavers (85%) provided high mortality of *Diaprepes abbreviatus* than when applied in aqueous suspension (42%). IJs in nematode-infected cadavers are reported to have better

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infectivity, dispersal, and tolerance to harsh environmental conditions than IJs applied in an aqueous suspension (Shapiro and Glazer 1996; Shapiro and Lewis 1999; Gulzar et al. 2020). It should be noted that **Chapter 4** had several limitations, and thus serves as a preliminary study for future research aimed at understanding the effects of application methods on the efficacy of EPNs. These limitations could not be addressed in the current study due to constraints of time and availability of the host insect but should be considered in future studies.

Future studies

The study compared the virulence of five EPN species against uncased pupae of *Gonipterus* sp. 2 in microwell plates and identified *S. yirgalemense* as the most virulent, and thus the suggested focus for further studies towards the development of EPNs to control *Gonipterus* sp. 2. The lethal concentration of *S. yirgalemense* against uncased pupae of *Gonipterus* sp. 2 was determined, which is important for informing the application rate in the field. The study also contributed towards knowledge on the host-foraging strategy of IJs of EPNs, although further studies are needed to clarify some aspects of the movement behaviour. These include the ability of EPNs to infect a sedentary host in deep soil and dispersal on a substrate with different textures. Other traits associated with the host-foraging strategy, such as nictation behaviour, jumping and efficacy against a mobile host, also require investigation.

The results from this study were from assays and experiments in a laboratory setting and further research to investigate the efficacy of the EPNs in the field is needed. EPNs may be highly virulent but still need to be applied such that they can access the habitat of the host and infect it. This study showed that application of EPNs with both infested cadavers and in an aqueous suspension can be effective, but that experiment needs to be repeated to confirm the results. Ultimately, the efficacy of application methods will need to be tested in the field. This will

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require testing different formulation and application methods and how they affect the behaviour of EPNs post-application.

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