# Entomopathogenic nematodes for the control of the vine mealybug (*Planococcus ficus*) in South African wine and table grapes

by
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## Declaration

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## Abstract

*Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), the vine mealybug, is of economic importance to the wine and table grape (viticulture) industries, as it characteristically causes more damage than other mealybug species. Mealybug infestations contaminate grapes with their waxy secretions, egg-sacs and honeydew production, on which sooty mould grows, resulting in the fruit being unmarketable. Many export grapes are rejected, prior to shipment, as a result of infestations and phytosanitary concerns with regard to mealybug infestations. They are also vectors for various plant viruses. Up to date, the most common method of mealybug control in South Africa has been the use of chemical insecticides. Unfortunately, mealybugs are difficult to control chemically, due to their secretive/hidden lifestyle, where chemicals do not reach them. Of great concern is the ability of mealybugs to rapidly build up resistance to insecticides as well as the negative environmental effects associated with chemical pesticide use. Alternatively, entomopathogenic nematodes (EPNs), belonging to the families Heterorhabditidae and Steinernematidae, have been identified as lethal insect pathogens and their insecticidal action, towards a variety of insect pests, has proven them to be valuable and effective biocontrol agents.

Laboratory bio-assays, to determine the ability of eight different EPN isolates to infect and kill *P. ficus,* were conducted. Six of the isolates were indigenous species and the other two, *Heterorhabditis bacteriophora* and *Steinernema feltiae,* were produced in Germany and are commercially available in South Africa. *Planococcus ficus* was highly susceptible to two indigenous species, *Heterorhabditis zealandica and Steinernema yirgalemense;* responsible for 96% ± 2% and 65% ± 10% mealybug mortalities, respectively. Biological studies illustrated that both *H. zealandica* and *S. yirgalemense* are able to complete their life cycles within adult female *P. ficus.* There was no significant difference in the pathogenicity of commercially produced *H. bacteriophora,* recycled through an insect host, and those from the formulated commercial product. However, commercially produced *S. feltiae* individuals, that were recycled through an insect host, were statistically more effective than those that were not. The $LC_{50}$ and $LC_{90}$ values for *H. zealandica,* in the current study, were 19 and 82 infective juveniles (IJs) respectively,

which were similar to the $LC_{50}$ and $LC_{90}$ values for *S. yirgalemense* at 13 and 80, respectively. The $LC_{50}$ and $LC_{90,}$ for commercially available *H. bacteriophora,* were greater than they were for both *H. zealandica* and *S. yirgalemense*, with values of 36 and 555, respectively. Such results indicate that there is a definite positive relationship which exists between the concentration of IJs of all three nematode species, used for inoculation, and the percentage mortality of *P. ficus.* Sand column tests resulted in *S. yirgalemense* outperforming *H. zealandica* significantly, with average mortalities of 95% ± 1.4% and 82% ± 4.1%, respectively. As a result *S. yirgalemense* was chosen for further studies in the field.

IJs of commercially produced *H. bacteriophora* and *S. feltiae* were exposed to imidacloprid in laboratory bioassays to determine the effect on survival and infectivity. This study established the fact that these two EPN species can be applied, in combination with imidacloprid, in an integrated pest management scheme. Soil application field trials at Welgevallen and Nietvoorbij, using *S. yirgalemense* and mealybugs in Eppendorf tubes, buried 15 cm in the soil, resulted in 50% ± 10% and 52% ± 12% mealybug mortalities, respectively, when applying IJs at a concentration of 80 IJs/$cm^2$. No significant difference was found between mealybug mortalities as a result of the three IJ concentrations applied (20, 40 and 80 IJs/$cm^2$) for both vineyards. Persistence trials indicated that after four months post application, *Cydia pomonella* larval mortalities showed no significant reduction in infectivity on the Welgevallen vineyard, while on the Nietvoorbij vineyard there were no larval mortalities.

Tests to establish whether or not *S. yirgalemense* and *H. zealandica* produced ant deterrent factors, showed no significant differences between the number of intact cadavers for both nematode species and for cadavers that were either four or six days old. There is, however, indication that deterrent factors may be in action in cadavers that were used six days after inoculation with 60% and 49% remaining intact for *H. zealandica* and *S. yirgalemense* infected cadavers respectively. All freeze killed cadavers were consumed by *Linepithema humile* (Mayr) (Argentine ant).

The effects of low temperatures on EPN movement and infectivity were tested for *H. zealandica* and *S. yirgalemense* in the laboratory. The mortality of *P. ficus* at 14˚C, as opposed to 25˚C, for *S. yirgalemense* and *H. zealandica* were found to be 9.1% ± 2.6% and 2.5% ± 1.2% respectively. Vertical sand column tests were also conducted at 14˚C for *S. yirgalemense* and *H. zealandica,* which produced

low mealybug mortalities of 3.5% ± 2.4% and 8.5% ± 1.4% respectively. This illustrates the low infectivity of the two local species at low temperatures. Laboratory persistence trials, conducted over a period of four months with *S. yirgalemense,* showed steady persistence of 100%, while *H. zealandica* had a statistically significant decrease of codling moth mortalities to 44% ± 5%.

A three armed olfactometer was designed to establish if *S. yirgalemense* responds and moves towards chemical cues in the soil. A significant greater average number of IJs moved towards the grape vine roots (246 ± 0.124 IJs), than to the mealybugs (133 ± 0.168 IJs) and to the control (4 ± 1.02 IJs). This demonstrates that *S. yirgalemense* does actively seek out its hosts and that volatile cues produced by damaged grape vine roots, are more attractive to the EPN than cues produced by *P. ficus*.

This study illustrates that *S. yirgalemense* has great potential as a biopesticide for controlling *P. ficus* in the soil of South African grape vineyards. Emphasis was placed on soil application of *S. yirgalemense* in the field, which produced good results, while laboratory tests indicate the potential for further aerial field application trials on grape vines. As the EPNs are not negatively affected by the agrochemical imidacloprid, the simultaneous use and combined action of both agents will potentially provide the farmer with excellent control against *P. ficus*. Further field- and aerial application studies will complement the current study and hopefully provide positive results for the efficient control of *P. ficus* found on grape vines.

# Opsomming

*Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), die wingerd witluis, is van groot ekonomiese belang vir die wyn en tafeldruif industrieë, aangesien dit kenmerkend meer skade veroorsaak as enige ander witluis spesies. Witluis infestasies besmet druiwe met hulle wasagtige afskeidings, eierssakke en heuningdou produksie, waarop swamme groei, wat tot gevolg het dat die druiwe onbemarkbaar is. Baie besendings druiwe, bestem vir uitvoer, word afgekeur weens witluis besmettings en ook as gevolg van fitosanitêre oorwegings. Hulle tree ook op as vektore van verskeie plantvirusse. Die mees algemene manier waarmee witluis in Suid-Afrika beheer word, is chemiese behandeling. Ongelukkig is witluis baie moeilik om chemies te beheer vanweë hulle verskuilde lewenswyse wat dit moeilik maak vir chemikalieë om hulle te bereik. Die vermoë van witluis om vinnig weerstand op te bou teen insekdoders, asook die negatiewe effek van chemiese middels op die omgewing, is kommerwekkend. Alternatiewelik, kan entomopatogeniese nematodes (EPNs) van die families Heterorhabditidae en Steinernematidae gebruik word vir die beheer van witluis. Hierdie nematodes is geïdentifiseer is as dodelike insek patogene, vir 'n groot verskeidenheid van pes insekte en daar is bewys dat hulle as waardevolle en effektiewe biologiese beheer agente kan optree.

Laboratorium biotoetse is gedoen om die vermoë van agt EPN isolate te evalueer om *P. ficus* te beheer. Ses van die EPN isolate is inheems, terwyl die ander twee, *Heterorhabditis bacteriophora* en *Steinernema feltiae,* in Duitsland produseer is en kommersieel beskikbaar is in Suid-Afrika. *Planococcus ficus* is hoogs vatbaar vir die twee inheemse EPN spesies, naamlik *Heterorhabditis zealandica* en *Steinernema yirgalemense* en hulle is verantwoordelik vir 96% ± 2% en 65% ± 10% van witluis mortaliteit. Biologiese studies het aangetoon dat beide *H. zealandica* en *S. yirgalemense* in staat is om hul lewensiklus te voltooi in volwasse wyfies van *P. ficus*. Daar is geen beduidende verskil gevind in die patogenisiteit van die geformuleerde kommersiële produk *H. bacteriophora* en dié wat *in vivo* geproduseer is nie. Daar is egter in die geval van *S. feltiae,* gevind dat die nematodes, wat in insekte produseer is, statisties beduidend meer effektief was, as dié wat kommersieel beskikbaar was. Die $LC_{50}$ en die $LC_{90}$ waardes van *H. zealandica,* in die huidige studie, was 19 en 82 infektiewe larwes (IJs), wat

baie naby die $LC_{50}$ en $LC_{90}$ waarders van *S. yirgalemense* van 13 en 80 was. Die $LC_{50}$ en $LC_{90}$ vir die kommersieel beskikbare *H. bacteriophora* was groter as vir beide *H. zealandica* en *S. yirgalemense*, met waardes van 36 en 555 onderskeidelik. Hierdie resultate dui daarop dat daar 'n positiewe verwantskap bestaan tussen die konsentrasie van die IJs van drie EPN spesies en die persentasie mortaliteit van *P. ficus*. Sand kolomtoetse dui daarop dat *S. yirgalemense* baie beter vaar as *H. zealandica* met gemiddelde mortaliteite van 95% ± 1.4% en 82% ± 4.1% onderskeidelik. Op grond van hierdie resultate is *S. yirgalemense* gebruik vir verdere veld studies.

IJs van kommersieel geproduseerde *H. bacteriophora* en *S. feltiae* is in laboratorium biotoetse blootgestel aan imidacloprid om die effek op die oorlewing en infektiewe vermoë vas te stel. Hierdie studie het aangetoon dat die twee EPN spesies aangewend kan word saam met imidacloprid in 'n geïntegreerde plaagbestuur opset.

Grond aanwendings is in veld proewe by Welgevallen en Nietvoorbij gedoen deur gebruik te maak van *S. yirgalemense* en *P. ficus* volwasse wyfies in Eppendorf buisies, 15 cm in die grond begrawe, het albei 50% ± 10% en 52% ± 10% witluis mortaliteit, respektiewelik, tot gevolg gehad, met die toediening van nematodes teen 'n konsentrasie van 80 IJs/$cm^2$. Geen beduidende verskille is gevind tussen die witluismortaliteit en die resultate van die verskillende EPN konsentrasies (20, 40 en 80 IJs/$cm^2$) wat op beide wingerde toegedien is nie. Oorlewings toetse het aangedui dat, drie maande na toediening, met *Cydia pomonella* as indikator, geen beduidende verskille in die infeksie potensiaal van die Welgevallen wingerd to gevolg gehad het nie, terwyl daar op die Nietvoorbij wingerd geen verdere larvale mortaliteit gevind is was nie.

Toetse om vas te stel of *S. yirgalemense* en *H. zealandica* afkrikmiddels vir miere in besmette insek kadawers produseer het aangetoon dat daar geen beduidende verskil is tussen die getal kadawers wat intakt is vir beide EPN spesies en kadawers wat vier of ses dae oud is nie. Daar is egter aangetoon dat die afskrikmiddels wel ses dae na infeksie deur insek kadawers afgeskei word; aangesien 60% en 49% van die oorblywende kadawers nog volledig was toe dit geïnfekteer was met *H. zealandica* en *S. yirgalemense*, onderskeidelik. Al die insek kadawers, wat deur bevriesing doodgemaak is, was deur *Linepithema humile* (Mayr) (Argentynse mier) verorber.

Die effek van lae temperature op EPN beweging en infeksie is vir *H. zealandica* en *S. yirgalemense* in die laboratorium getoets. Die mortaliteit vir *P. ficus* by 14°C in vergelyking met 25°C vir *S. yirgalemense* en *H. bacteriophora* is onderskeidelik as 9.1% ± 2.6% en 2.5% ± 1.2% gevind. Vertikale sand kolom toetse is ook uitgevoer by 14°C vir *S. yirgalemense* en *H. zealandica* het baie lae witluis infeksie van 3.5% ± 2.4% en 8.5% ± 1.4% respektiewelik, veroorsaak. Dit illustreer die lae infeksie potensiaal van die twee lokale nematode spesies by lae temperature. Die nawerking van *S. yirgalemense* en *H. zealandica* is oor 'n periode van vier maande in die laboratorium vasgestel. Volhoubare nawerking van 100% is met *S. yirgalemense* na verloop van vier maande verkry, terwyl daar in die geval van *H. zealandica* daar 'n beduidende afname van tot 44% ± 5% in kodling mot larvale mortaliteit was.

Laastens is daar 'n loktoets opgestel, deur 'n drie-arm olfaktometer te ontwerp, om vas te stel of *S. yirgalemense* reageer en aangelok word deur chemiese seine in die grond. Daar is demonstreer dat 'n beduidende groter getal nematodes het na die wingerdwortels (246 ± 0.124 IJs), as na die *P. ficus* volwasse wyfies (133 ± 0.168 IJs), as na die kontrole (4 ± 1.02 IJs) beweeg. Dit demonstreer dat *S. yirgalemense* aktief sy gasheer opspoor deur gebruik te maak van chemiese seine en dat seine wat deur beskadigde wingerdwortels geproduseer word, meer aanloklik vir die nematodes is as dié chemiese seine wat deur die witluise self afgeskei word.

Hierdie studie illustreer die groot potensiaal van *S. yirgalemense* as 'n biologiese beheer agente vir die beheer van *P. ficus* in wingerde grond in Suid-Afrika. Klem is gelê op grond toediening van *S. yirgalemense*, wat goeie resultate geproduseer het, terwyl laboratorium toetse aangetoon het dat daar potensiaal is vir verdere navorsing met bogrondse aanwending van nematodes in wingerd. Aangesien EPNs nie negatief affekteer word deur die landbou chemikalie, imidacloprid nie, kan die twee gesamentlik aangewend word en die gekombineerde aksie van beide produkte het die potensiaal om die produsent uitstekende beheer van *P. ficus* te bied. Verdere navorsing op grond en bogrondse nematode aanwending kan aansluit by die huidige studie en sal hopelik positiewe resultate lewer vir die effektiewe beheer van die *P. ficus* populasie in wingerd.

# Table of Contents

# List of Figures

## List of Tables

# CHAPTER 1

## An Overview of the Vine Mealybug (*Planococcus ficus*) in South African Vineyards and the use of Entomopathogenic Nematodes as Potential Biocontrol Agent

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### Vine Mealybug: *Planococcus ficus* (Signoret)

### Introduction

The mealybug family (Hemiptera: Pseudococcidae) is large, consisting of more than 2240 recorded and described species that are categorised almost 300 genera (Millar, 2002; Ben-Dov & Miller, 2012). In South Africa, a total of 50 genera and 109 species have been recorded, of which 13 genera and 68 species are indigenous (Millar, 2002).

The general common name ‘mealybug’ is derived from the white mealy or powdery wax that is secreted to cover their bodies (Millar, 2002; Franco *et al*., 2009). Mealybugs are all phytophagous, with vine mealybugs being specifically phloem feeders (Millar, 2002; Daane *et al*., 2006). They are very small, soft-bodied insects with piercing, sap-sucking mouthparts. They encourage the growth of sooty mould on vines and grapes by producing a substrate of sticky honeydew (Millar, 2002; Franco *et al*., 2009). Mealybugs are considered severe agricultural pests, as their presence and feeding causes direct damage to plants by lowering production and rendering fruit unmarketable, while also transmitting various plant viruses (Greiger & Daane, 2001; Holm, 2008). Various studies have shown *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) to be the dominant mealybug species in South African vineyards, highlighting the demand for attention and need for control (Kriegler, 1954; Walton & Pringle, 2004b).

**History and Geographical Distribution**

The vine mealybug, *P. ficus*, has been subjected to repeated renaming, misidentification and reclassification in the past, due to the lack of qualitative characteristics to help distinguish it from other similar species (Walton, 2003). Currently, there are still various colloquial names in use that could easily lead to confusion. For instance, common names given to the species by De Villiers (2006) include both vine mealybug and grapevine mealybug, whereas Walton and Pringle (2004b) and Holm (2008) both provide another two vernacular names, the subterranean vine mealybug and the Mediterranean vine mealybug. From henceforth in the current thesis, the name, vine mealybug, will be used, keeping in mind that it must not be mistaken for the closely-related species, the grape mealybug, *Pseudococcus maritimus* (Ehrhorn).

The first South African vine mealybug sighting was recorded in 1914 (Holm, 2008). The mealybug in question was originally identified as *Planococcus citri* (Risso) and subsequently correctly identified as *P. ficus* in 1975 (Walton & Pringle, 2004b; Holm, 2008). *Planococcus ficus*, which was first recorded as being a problem in the Western Cape province vineyards in 1930, by 1935 had spread to the Hex River Valley and to all other major vineyards in the Western Cape (Walton, 2003; Walton & Pringle, 2004b; De Villiers, 2006; Holm, 2008). It is currently regarded as a key pest insect of the South African table grape and wine industries. Both the introduction of *P. ficus* to South African agriculture and its origins are uncertain. Presumably being native to the Mediterranean region, it is assumed to have entered the South African system via plant material (Walton & Pringle, 2004b; De Villiers, 2006). Internationally, it has spread and caused damage to vineyards in the Middle East, Pakistan, South America, California, the Mediterranean region, Mexico, Europe and North Africa, among other areas (De Villiers, 2006; De Villiers & Pringle, 2007; Daane *et al*., 2008).

**Morphology and Life Cycle**

Mealybug species are difficult to distinguish due to their close resemblance to one another and the lack of morphological descriptions in earlier studies. For example, there are only minor differences in the arrangement and number of glandular ducts on the dermis of *P. ficus* and *P. citri* (Walton, 2003; Walton &

Pringle, 2004b). Efforts have been made to assist with mealybug identification. Millar (2002) has provided a key to help identify South African Pseudococcidae genera, while other authors, such as Wakgari and Giliomee (2005), have developed a detailed diagnostic key, including morphometric characters, to distinguish the six mealybug species found on citrus in South Africa. Mealybug taxonomy is mostly based on the female anatomy, due to the short-lived, inconspicuous nature of the males (Millar, 2002; Holm, 2008). Millar (2002) expresses concern that, despite previous descriptive work already having been done, the identification of South African mealybugs, especially when they are in their nymphal or egg phases, still remains a challenge. Despite the difficulties experienced with morphological descriptions and identifications, molecular identification techniques have been developed to distinguish accurately between the different mealybug species. A molecular identification approach was successfully developed by Pieterse *et al*. (2010) to identify any life stage of the seven most important mealybugs found on citrus in South Africa, to species level. In North America, a multiplex PCR molecular tool was developed by Daane *et al*. (2011) to identify seven different problematic mealybug species found in vineyards. Of the seven species concerned, individuals such as *P*. *ficus*, *P*. *citri*, *Pseudococcus viburni* (Signoret) (obscure mealybug) and *Pseudococcus longispinus* (Targioni-Tozzetti) (longtailed mealybug) are common pests in South African vineyards.

*Females*

Vine mealybugs, like most scale insects, are sexually dimorphic (Holm, 2008; Franco *et al*., 2009). Females are neotenic and wingless, weighing about 100-200 times more than the adult male (Holm, 2008). Adult female *P. ficus* are approximately 4mm in length, slightly wider than 2mm and approximately 1.5mm thick. They are segmented, with a pink to slate-grey-coloured flesh that is covered by a fine white powdery wax layer. The fringe of the body has waxy hair-like extensions, while a thin dark line denuded of wax runs down the back of the body (Fig. 1.1).


Fig. 1.1: Female *Planococccus ficus* colony

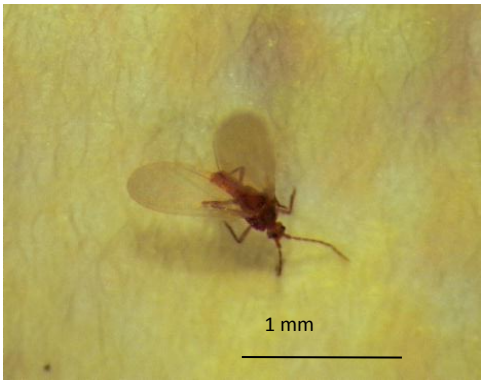


Fig.1.2: Adult male *Planococcus ficus.*

The female mealybug undergoes incomplete metamorphosis passing through five growth stages, including an egg, three nymphal instars (crawlers) and, lastly, the adult (Picker *et al*., 2002; Walton & Pringle, 2004b; Holm, 2008; Franco *et al*., 2009). After mating, each oviparous female lays an average of 362 eggs within white egg sacs or ovisacs constituted of filamentous waxy hairs (Walton, 2003).

*Males*

Male *P. ficus* adults are small, delicate, dipterous insects (Franco *et al*., 2009). They are less than 1mm long and brownish in colour, with a pair of inconspicuous, transparent wings. They have beaded antennae with a thorax wider than is the abdomen (Dreves & Walton, 2010). *Planococcus ficus* males have two long tail filaments (anal seta) to help with flight stabilisation, and no functional mouth parts. They have a short life span, with the single purpose of copulating with females who, at sexual maturity, release pheromones to attract the males (Walton & Pringle, 2004b; Franco *et al*., 2009; Dreves & Walton, 2010) (Fig. 1.2).

Male mealybugs go through complete metamorphosis, whereby distinguishing male characteristics become apparent after the third growth stage (Walton, 2003; Holm, 2008). Contrary to the female's five growth stages, males endure seven stages which include egg, three nymphal instars, pre-pupa, pupa and, lastly, the adult stage (Walton, 2003). *Planococcus ficus*, unlike *P. maritimus*, does not diapause through winter, resulting in all life stages being found in any given season, with populations in South Africa experiencing about five to six generations in any one year (Kriegler, 1954; Holm, 2008; Cid *et al*.,

2010). The variations in generation numbers that have been observed in other countries are held by Walton and Pringle (2004b) to possibly be related to the mean temperature differences involved.

**Host Plant Range**

*Planococcus ficus* is a polyphagous insect that feeds on a wide range of host plants apart from *Vitis vinifera* (grape vine) (Daane *et al*., 2008; Walton *et al*., 2009). Such feeding habits enable the provision of an unwanted source population of the pest outside vineyards (Haviland *et al*., 2005). In California, *P. ficus* has been reported to be found on subtropical and tropical crops, along with a few common weeds, whereas in Europe it is commonly found on fig trees (*Ficus* spp.) (Haviland *et al*., 2005).

*Seasonal distribution and phenological trends*

The vine mealybug displays a clear pattern of vertical seasonal movement on grape vines (De Villiers, 2006). The largest portion of the population has been found above ground, while their presence has also been found on vine roots, down to a depth of 30cm (Walton & Pringle, 2004a). *Planococcus ficus* colonies consist of overlapping generations, resulting in all stages of the life cycle being present at any time of the year (Walton, 2007; Holm, 2008). Population development and vertical movement through the course of the seasons is affected by the absence or presence of natural enemies, temperature and the availability of food (Walton, 2003; Holm, 2008).

Depending on which hemisphere one refers to, the seasonal movement of vine mealybugs is generally similar within both the northern and southern hemisphere. Populations follow similar trends that correspond to the progression of winter and summer. In South Africa, the vine mealybugs spend the winter months in colonies on lower regions of the plant, under the bark and underground (De Villiers, 2006; Holm, 2008). The upward movement of *P. ficus* on the vine trunk begins from spring to early summer (October/November) in the southern hemisphere and in March/April in such northern hemisphere countries as Italy and Israel (Walton, 2003; Walton & Pringle, 2004b). In both the Coachella and San Joaquin valleys of California, the upward vertical movement of mealybugs correlates with the warmer temperatures experienced during the summer months (Daane *et al*., 2003). So, regardless of in which hemisphere they occur, their upward movement shows clear correspondence with the onset of the

warmer summer months. Preceding such upward movement, *P. ficus* begins forming new colonies at the bases of young buds and shoots (De Villiers, 2006; Holm, 2008). From this point they move to the leaves and by December they are predominantly found feeding on whatever the foliage is available (De Villiers, 2006; Holm, 2008). Peak populations have been observed between January and the beginning of February, when the mealybug is found infesting grape bunches, where they feed on the abundant plant sap and on the available nutrients (Walton, 2003; Holm, 2008). Conversely, the lowest population levels on the aerial parts of the plant have been recorded during winter months (Walton, 2003). During harvest, many colonies are removed and after harvest (in autumn) they return to the leaves to feed and continue their migration back under the bark of the stems and trunk, where they overwinter (Walton & Pringle, 2004b; De Villiers, 2006; Holm, 2008).

Clearly, slight variations in peak and lowest population numbers and movement times occur from year to year. In the Hex River Valley, during the 2002/2003 season, De Villiers and Pringle (2007) observed peak *P. ficus* infestations in March as opposed to such infestations that were found between the end of January and the beginning of February by Walton and Pringle (2004b). Walton (2003) recorded the percentage infestation from 1999 to 2001 in the Hex River, Stellenbosch and Robertson areas, showing that peak infestations across the three areas could occur anywhere between mid-February and March. Such variations are generally due to differing temperatures, with, for example, cool early summer temperatures delaying the upward migration of the colonies and hence resulting in a delayed population peak (Walton & Pringle, 2004b).

Despite the general seasonal movement, the largest portion of the *P. ficus* population tends to occur on the vine trunk throughout the year (Walton, 2003; Walton, 2007). A preference for the trunk and woody branches has been observed by Walton (2007) in Stellenbosch, McGregor and Robertson in the Western Cape, with similar findings having been recorded by Cid *et al*. (2010) in Galician vineyards in north-western Spain. Both Walton (2007) and Cid *et al*. (2010) explain that the woody sections of the vine have bark layers that provide micro-habitats, giving *P. ficus* refuge and protection from natural enemies, extreme temperatures and insecticidal sprays. In addition, Walton (2007) states that old canes and trunks

are also much less disturbed during harvest and pruning, while, additionally, the phloem of the trunk is easily and consistently accessible to the mealybugs.

*Dispersal*

Mealybugs have a limited ability to move and to disperse, as females are wingless, with their movement being restricted to only minor distances (Holm, 2008). Female crawlers (first-instar nymphs) and adult males are mostly mobile and display dispersal activity. Immobility of female adults sets in when old individuals experience the deterioration and loss of their legs (Franco *et al*., 2009).

Poor pruning and harvesting techniques, along with the distribution of fruit, rootstock and grafting material, are responsible for the long-distance dispersal of *P. ficus* (Holm, 2008). Other shorter-distance dispersal mechanisms include adhering to wild and domestic animals, moving water and wind (Holm, 2008; Franco *et al*., 2009). Distribution is mostly aggregative, as crawlers mostly tend to settle close to the adult females on the natal host plant (Franco *et al*., 2009).

**Economic Importance**

South Africa is the second largest table grape producer to Chile in the southern hemisphere (De Villiers & Pringle, 2007). More than 80% of South African table grape production occurs in the Western Cape (Walton *et al*., 2009). The Eastern and Northern Cape, Mpumalanga, Limpopo and the Free State also produce grapes (Walton *et al*., 2009). For the 2011/2012 season, South Africa produced a total of 54.657 million cartons (4.5kg carton) of table grapes (SATI, 2012). In 2011, South Africa was ranked eighth in the world for the total volume production of liquid from grapes, with a total volume of 1012.8 million litres being split between wine, brandy, distilled wine and grape juice production (WOSA, 2012).

Mealybugs are pests of serious economic importance, infesting various fruit crops and ornamental plants around the world (Wakgari & Giliomee, 2003). The grape mealybug, *P. maritimus* and *P. ficus*, are two key pest species that cause great economic losses in South African, Californian, Spanish, Pakistani and South American vineyards (Greiger *et al*., 2001; De Villiers & Pringle, 2007; Cid *et al*., 2010).

Mealybug infestations contaminate grapes. Their waxy secretions, egg-sacs and honeydew production, on which sooty mould grows, result in the fruit being unmarketable, as the tolerance levels for cosmetic damage in the table grape industry are very low (Greiger & Daane, 2001; De Villiers & Pringle, 2007; Holm, 2008). Many consignments are rejected prior to shipment as a result of infestations and phytosanitary concerns. The market also has legislative restrictions for the presence of insecticidal residues on fruits, making the management of such pests increasingly more complicated (De Villiers & Pringle, 2007; Walton *et al*., 2009).

Serious mealybug infestations are able to inhibit the normal ripening process of grapes, causing poor taste and colour, and leading to the eventual withering of grape bunches (De Villiers, 2006; De Villiers & Pringle, 2007). Yellowing of leaves, premature leaf drop, weakening of the vine, decreased vigour and lifespan might also occur, due to excessive feeding of the mealybug (De Villiers, 2006; De Villiers & Pringle, 2007; Holm, 2008).

*Planococcus ficus* has characteristics that make it particularly more economically damaging than other mealybug species (Haviland *et al*., 2005; Daane *et al*., 2008). Compared to *P. maritimus, P. longispinus* and *P. viburni*, *P. ficus* excretes far more honeydew per individual, and has a faster development time and a higher reproductive rate of more than 250 eggs produced per female. In addition to feeding on all parts of the vine throughout the season, they have a wider host range than the other mealybug species (Daane *et al*., 2003; Haviland *et al*., 2005; Daane *et al*., 2008).

Finally, *P. ficus* is a viral disease vector, which renders it a problem even when the pest occurs at low densities (Haviland *et al*., 2005; Holm, 2008). The vine mealybug, along with *P. longispinus and P. viburni*, are all vectors of the grapevine leafroll-associated virus 3 (GLRaV-3) (Petersen & Charles, 1997; Walton & Pringle, 2004b). GLRaV-3 reduces the amount of photosynthesis that takes place, thus reducing the quality and yield of grapes by delaying sugar accumulation and ripening while increasing acidity levels of the grapes, making it an economically important disease of *V. vinifera* (Petersen & Charles, 1997; Carstens, 2002; Walton & Pringle, 2004b). Symptoms of GLRaV-3 vary, depending on the cultivar grown, as well as on the differing environmental conditions that prevail (Carstens, 2002). Leaves generally show symptoms of downward-rolled margins, green veins and red interneural discolouration

(Carstens, 2002; Douglas & Kruger, 2008). More so, *P. ficus* is a virus vector of both Shiraz and corky-bark diseases (Walton & Pringle, 2004b; Holm, 2008).

**Relationships with Ants**

The trophobiotic relationship between mealybugs and honeydew-seeking ants requires attention. This relationship is one in which ants obtain carbohydrate-rich honeydew from the mealybug while providing it with protection, transport and sanitation in exchange (Mgocheki & Addison, 2009). Thus, ants are able to exacerbate mealybug pest problems by disrupting processes of augmentative and natural biological control and by aiding in their dispersal (Phillips & Sherk, 1991; Daane *et al*., 2008). In the presence of ants, mealybugs are able to consume larger quantities of plant sap than they otherwise would. Moreover, some ant species, such as the cocktail ant, *Crematogaster peringueyi* (Emery), actually construct a shelter over *P. ficus* in order to provide it with protection (Franco *et al*., 2009; Mgocheki & Addison, 2009). The mutualistic relationship concerned has been shown to significantly reduce the efficacy of biological control of *P. ficus* (Addison, 2002). The most common pest ant species in South African vineyards include *Linepithema humile* (Mayr) (Argentine ant), *Anoplolepis custodiens* (Smith) (common pugnacious ant) and *Anoplolepis steingroeveri* (Forel) (black pugnacious ant), which protect mealybugs from parasitoids such as *Coccidoxenoides perminutus* (Timberlake) (Addison, 2002).

Due to the above factors it is, thus, important that ants, too, are controlled to help enhance the effectiveness of biocontrol. Such control is currently being performed by means of the use of chemical pesticides in the form of chemical stem barriers (Mgocheki, 2008). In comparison to other methods, stem barriers have been found to be the most effective against various ant pests (Addison, 2002).

**Control and Monitoring Options**

*Chemical control*

Up to date, the most common method of mealybug pest control in South Africa has been the use of chemical insecticides. Both short residual organophosphates (e.g. mevinphos, applied during the growing season) and long residual organophosphates (e.g. chlorpyrifos, applied just before bud break in late

August) are commonly used (Walton *et al*., 2004; Daane *et al*., 2006; Holm, 2008). Unfortunately, mealybugs are difficult to control chemically, due to their cryptic lifestyle, which involves hiding in crevices, under the bark and on the roots, where chemicals do not reach them (Walton & Pringle, 2004b). Mealybugs are covered by their typical hydrophobic waxy secretions, which serves to repel any water-based insecticide solutions (Franco *et al*., 2009). More concerning is the ability of mealybugs to rapidly build up resistance to insecticides (Flaherty *et al*., 1982; Walton & Pringle, 2004b; Franco *et al*., 2009). The lack of selectivity of such pesticides ultimately causes an increase in pest densities. By killing natural predators, the pesticides reduce the levels of natural biocontrol agents (Wakgari & Giliomee, 2003; Daane *et al*., 2006; Holm, 2008). As Mgocheki (2008) states, pesticides often kill off more of the natural enemy populations than of the intended pest, allowing the pest populations concerned to recover and, in some cases, causing a secondary pest outbreak of a species that previously was not a problem.

*Biological control*

Classic biological control generally involves the release of an exotic, natural enemy in order to reduce and control population numbers of an introduced pest species, with the intended permanent establishment of the biological control agent (Gaugler *et al*., 1997; Van Lenteren *et al*., 2003). In contrast, inundative biological control is when large amounts of the biological control agent are released with the intention of reducing the pest population in the absence of the establishment and continuing effects of the biological control agent (Van Lenteren *et al*., 2003). The most prevalent natural enemies of *P. ficus* in South Africa include such Hymenopteran parasites as *C. perminutus* (which is commercially produced and available for augmentative release), *Anagyrus pseudococci* (Girault), *Leptomastix dactylopii* (Howard) and such predatory Coccinellid beetles as *Nephus bineavatus* (Mulsant), *N. quadrivittatus* (Mulsant) and *N. angustus* (Casey) (Wakgari & Giliomee, 2003; Walton & Pringle, 2004a, 2004b; Holm, 2008; Mgocheki & Addison, 2009). Unfortunately, parasitoids can only attack mealybugs when they are found on exposed locations, thus they are unable to reach *P. ficus* when it is underground or hiding beneath bark or in deep crevices (Holm, 2008). To exacerbate the problem, ants interfere with parasitism and reduce parasitoid numbers by directly killing individuals (Mgocheki & Addison, 2009). Biological

control has been the primary alternative to chemical pesticide, and is considered one of the cornerstones in integrated pest management (IPM) schemes (Gaugler *et al.,* 1997; Koppenhöfer *et al*., 2000).

*Cultural control*

Cultural methods of control are generally designed to reduce the spread of existing mealybug infestations to uninfested vineyards (Holm, 2008). Such reduction in spread can be effected by means of organising and coordinating the on-farm movement of labourers, tools and machinery (Walton & Pringle, 2004b). The sterilisation of harvesting and pruning equipment is very important to reduce contamination (Holm, 2008). Correct summer pruning and the removal of dead and/or excess twigs, branches and leaves increases the effectivity of insecticides, predators and parasitoids (Walton & Pringle, 2004b).The preservation of natural surrounding vegetation is important to provide a source for natural enemies of *P. ficus* and other agricultural pests, while increasing the biodiversity of the agro-ecosystem concerned (Bowler, 2002; Walton & Pringle, 2004b).

*Integrated pest management (IPM)*

Unfortunately, chemical control has proved itself incapable of ensuring 100% control of *P. ficus*, while no cultural control method can totally prevent infestations (Walton, 2007). The increasing strictness in terms of export requirements concerning insecticide residues on produce, highlight the need for a truly effective IPM control system (Walton & Pringle, 1999). According to both Bowler (2002) and Pretty *et al.* (1995), IPM uses a combination of various pest control methods to try to reduce pest populations in a sustainable, non-polluting way. IPM strategies, which can be highly effective if they are administered correctly, are recommended for the control of *P. ficus* (Walton & Pringle, 2004b; Holm, 2008). Although an IPM system should complement biological control methods, the poor implementation of a single strategy can easily have negative effects on the entire IPM programme, which, in reality, is often the case (Walton & Pringle, 2004b; Holm, 2008). Wakgari and Giliomee (2003) mention that, for IPM strategies to be successful, a degree of knowledge of the mortality levels exerted by current natural enemies, of the density and spatial interactions of natural enemies, and of the effects of other control methods on the pest

species needs to be known. The execution of tests is also required to ensure the compatibility of the various control methods used in combination in the IPM system.

*Monitoring*

Monitoring is essential for the successful control of a key pest species, such as *P. ficus*, as it provides valuable information regarding the pest's density and presence for consultants to select the best management options (Walton *et al*., 2004; De Villiers, 2006; Daane *et al*., 2008). Direct measures, such as the visual sampling of *P. ficus* in the vineyard, is a difficult, timely and labour-intensive process, which is only effective in late summer, by which time crop damage would have already occurred (Walton *et al.*, 2004; Franco *et al*., 2009). As a result, a more effective monitoring system that allows for detection early in the season is needed.

A relative monitoring system using sex pheromone-based traps has proven to be a more effective mealybug colony detection tool and early warning system compared to other systems (Walton *et al.*, 2004; Daane *et al*., 2006; Dreves & Walton, 2010). Female mealybugs emit species-specific sex pheromones to attract males for mating (Daane *et al*., 2006; Franco *et al*., 2009). The pheromone concerned is non-toxic and effective in very small quantities when used in pheromone traps, making for far easier and more efficient capture of males than the manual searching for cryptic females would be (Franco *et al*., 2009; Dreves & Walton, 2010). The *P. ficus* sex hormone is lavandulyl senecioate, a monterpene ester, which has been synthetically produced and tested as a monitoring tool by Millar *et al*. (2002) in Californian vineyards and by Walton *et al*. (2004) in South African vineyards. Sex pheromones can also be effectively used not only as a monitoring tool, but also as a means of population control and reduction by means of the mass trapping of males and/or as a means of mating disruption (Daane *et al*., 2006; Franco *et al*., 2009).

## Entomopathogenic Nematodes

### Introduction

Entomopathogenic nematodes (EPNs) have been known about since the 17th century, but serious attention has only been given to using nematodes for insect control since the 1930s (Smart, 1995). Interest in EPNs was initiated in 1929 when Glaser and Fox found grubs of the Japanese beetle, *Popillia japonica* (Glaser), infected with the nematode *Steinernema glaseri* Steiner, 1929 (Smart, 1995; Ehlers, 2001). With the increasing development of effective, cheap chemical pesticides from the 1940s to the 1960s, the work and discoveries of Glaser took a back seat until recently (Smart, 1995; Adams & Nguyen, 2002). Since the negative environmental effects, decreasing affectivity, and increase in cost of chemicals became apparent in the mid-1960s, there has been an increasing need to find biological alternatives in terms of insect pest management (Smart, 1995; Adams & Nguyen, 2002). Subsequently many new nematode species with biocontrol potential have been discovered, described and tested over the past decade (Adams & Nguyen, 2002; Stock & Hunt, 2005). The proof of such efforts is evident, with Stock and Hunt (2005) providing a key to, and the morphological diagnosis of, 11 different nematode families used in biocontrol, of which over 100 nematode species are mentioned and described. EPNs belonging to the families Heterorhabditidae and Steinernematidae are deadly insect pathogens that play a role in the regulation of natural insect population levels, mostly in the soil (Griffin *et al*., 2005; Kaya *et al*., 1993). Of particular interest regarding the two families of EPNs concerned is their inundative application as a biocontrol agent for economically important insect pests (Griffin *et al*., 2005).

### Biology and Life Cycle

Heterorhabditids and Steinernematids both progress though four immature stages before reaching maturity (Adams & Nguyen, 2002). In both families, the third stage has a free-living, non-feeding infective juvenile (IJ) or dauer (which is German for ‘enduring’) juvenile. The IJ is well adapted to long-term survival in the soil while waiting for, or seeking out, a host (Ehlers, 2001).

The two families differ in their modes of reproduction, such that, in the first generation of Heterorhabditidae, there are only hermaphrodites, while males and females are produced in proceeding

generations (Griffin *et al*., 2005). In contrast, all Steinernematid generations are amphimictic (Griffin *et al*., 2005). Heterorhabditids and steinernematids both have obligatory symbiotic associations with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Boemare, 2001; Ehlers, 2001; Griffin *et al*., 2005). *Photorhabdus* and *Xenorhabdus* are both gram-negative bacteria belonging to the family Enterobacteriaceae (Boemare, 2001). Steinernematid IJs retain *Xenorhabdus* symbionts within an intestinal vesicle, while *Photorhabdus* cells stick together in the anterior part of Heterorhabditis's gut (Boemare, 2001).

When encountering a suitable insect host, the IJ enters via natural openings such as the anus, mouth or spiracles (Gaugler *et al*., 1997; Griffin *et al*., 2005). Heterorhabditis can bore directly into the haemocoel through thin parts of the cuticle, by means of an anterior dorsal tooth (Gaugler *et al*., 1997; Griffin *et al*., 2005). Once in the insect's haemocoel, the IJ experiences a process called 'recovery', whereby the bacterial symbionts from their gut are released (Ehlers, 2001; Griffin *et al*., 2005). The bacteria grow rapidly within the nutrient-rich haemolymph, while producing toxins and other metabolites that kill off the host within 24 to 48 hours after infection (Gaugler *et al*., 1997; Ehlers, 2001; Griffin *et al*., 2005). The bacteria also produce antimicrobial compounds that prevent the development of any other microbes within the cadaver, resulting in a monoxenic microcosm (Boemare, 2001). The nematodes then change into J3 juveniles, which feed on the symbiotic bacteria as well as on host tissue that is broken down by the bacteria. Subsequently the development of the J4 occurs, which then develops into adults of the first generation (Ehlers, 2001; Adams & Nguyen, 2002). Once the adults concerned mate, the females lay eggs that hatch and moult successively through four stages, of which the fourth stage develops into adults. The process continues in this way as long as the insect cadaver supplies sufficient resources (Ehlers, 2001; Adams & Nguyen, 2002). Such insect cadavers normally allow for the development approximately two or three EPN generations. Once resources are depleted, the offspring develop into third-stage IJs, which stop feeding and incorporate the symbiotic bacteria before exiting the cadaver in search of a new host (Ehlers, 2001; Adams & Nguyen, 2002). IJs are, however, able to survive in the soil for several months without a host (Adams & Nguyen, 2002).

**Compatibility of Nematodes with Agrochemicals**

In an IPM system, an important factor to consider is the compatibility and interactions of EPNs with various agrochemicals (García del Pino & Jové, 2005; Gutiérrez *et al*., 2008). It would be advantageous to know whether such agrochemicals as pesticides could be applied simultaneously or tank-mixed with EPNs in order to save both money and time while facilitating the EPNs in an IPM system (De Nardo & Grewal, 2003; Koppenhöfer & Grewal, 2005).

Many studies have been done on the effects of chemicals on such EPN species as *Steinernema feltiae* Filipjev 1934, *Steinernema carpocapsae* Weiser 1955 and *Heterorhabditis bacteriophora* Poinar 1976 (Rovesti & Deseö, 1990; Head *et al*., 2000; Koppenhöfer *et al*., 2000; De Nardo & Grewal, 2003; Alumai & Grewal, 2004; García del Pino & Jové, 2005; Gutiérrez *et al*., 2008). The compatibility of two endemic nematodes, *Heterorhabditis zealandica* Poinar 1990 and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 were tested with aqueous solutions of two adjuvants (Nu-Film-P® and Zeba®), two biopesticides (Helicovir™ and Cryptogran™) and one insecticide (Cyperphos 500 E.C.®) by Van Niekerk (2012). Results showed that both species were compatible with the chemicals showing no significant reduction in levels of IJ infectivity. However, *S. yirgalemense* did show a significant increase in mortality after being exposed to the various chemicals concerned. In contrast it has been found in many other cases that IJs are compatible and that they show a relative insensitivity to a variety of different chemical formulations (García del Pino & Jové, 2005). Thus, different agrochemicals affect different EPN species in various ways, such that the way in which each species is affected requires evaluation (Koppenhöfer & Grewal, 2005). The different effects on IJs have been shown to be either synergistic (additive), negative in terms of IJ infectivity and persistence, or to have no effect at all on the nematode (Koppenhöfer *et al*., 2000; Koppenhöfer & Grewal, 2005).

Koppenhöfer and Grewal (2005) recommended that incompatible EPNs and agrochemicals can be managed by choosing an appropriate time interval between the applications of the two agents, depending on the persistence of the chemical concerned. A period of 1 to 2 weeks is generally recommended after a chemical application before EPNs are applied (Koppenhöfer & Grewal, 2005). Head *et al*. (2000) tested not only the direct effects of insecticides on *S. feltiae* but also the effects of foliar chemical residues on

the IJs. Results showed that sequential, instead of simultaneous, application of EPNs and agrichemicals might often be the better choice in an IPM system.

## Application

*Above ground*

The use and application of EPNs has traditionally been focused on, and they are considered most suited to the control of soil-dwelling insect pests and/or the soil stages of insect life cycles (Wilson & Gaugler, 2004). Unfortunately, the commercial use of nematodes for above-ground pests has mostly been unsuccessful and plagued with problems (Lello *et al*., 1996; Shapiro-Ilan *et al*., 2006). The sensitivity of the IJs on exposed surfaces has left above-ground treatments highly dependent on the prevailing weather conditions, resulting in discouragingly erratic results (Gaugler, 1988). However, tests have demonstrated the potential of aerial application with certain EPN species against particular insect pests (Lello *et al*., 1996; Shapiro-Ilan *et al*., 2006; De Waal, 2008). Targeting insects living in above-ground cryptic habitats shields the IJs from lethal environmental factors, enabling some promising results (Mason *et al*., 1999; Shapiro-Ilan *et al*., 2006). Failures can mostly be attributed to the IJs' sensitivity to abiotic factors, including ultraviolet (UV) radiation, desiccation and extreme temperatures (Smits, 1996; Mason *et al*., 1998). Subsequently, the successful control of above-ground insect pests using nematodes is a challenge, when considering the unfavourable aerial conditions.

Suggestions have been made to help minimise the negative environmental effects of foliar application. As temperatures below 0°C and above 40°C are lethal to most EPNs, the water temperature used for application should fall within the 4-30°C range (Smits, 1996; Wright *et al*., 2005). To avoid desiccation and to reduce the effects of the sun's UV rays, EPNs can be applied early in the morning (when there is an added bonus chance of dew) or just prior to dusk (Lello *et al*., 1996; Mason *et al*., 1999). Adjuvants can be added to the spray solution used. Adjuvants have been found to help reduce impacts of desiccation and water surface tensions, allowing IJs to move out of discrete spray droplets, thus increasing the number of infecting nematodes (Mason *et al*., 1999; Gan-Mor & Matthews, 2003). Van Niekerk (2012) tested the effects that two adjuvants, Nu-Film-P® (a spreader or sticker) and Zeba® (an

anti-desiccant), may have on *H. zealandica* and *S. yirgalemense* survival and infectivity. The study showed that Zeba® significantly improved the ability of *S. yirgalemense* to infect and kill *P. citri* by reducing the negative effects of desiccation in the laboratory.

Another area of application that requires consideration is the technology used. Suboptimal application methods have contributed significantly to the failure of aerial treatments (Mason *et al*., 1998, 1999). Different spray and spinning disc technologies have been tested, while considerations of nozzle and pump types, droplet size, spray distribution and spray pressures have been undertaken to enhance the success rate of aerial nematode application (Lello *et al*., 1996; Mason *et al*., 1998, 1999; Gan-Mor & Matthews, 2003; Shapiro-Ilan *et al*., 2006).

*Soil*

Although the soil is the natural habitat for nematodes, and is a more suitable target habitat, many attempts to control insects within the soil have failed (Kung *et al*., 1990; Shapiro-Ilan *et al*., 2006). Just as with aerial application, many biotic, abiotic and application technology factors need to be considered. Abiotic factors such as soil moisture (of which too much can restrict nematode movement and cause oxygen deprivation), temperature and relative humidity have varying effects on the pathogenicity and survival of different nematode species (Kung *et al*., 1991; Shapiro-Ilan *et al*., 2006). Equally, different soil textures (sand, sandy loam, clay loam and clay) have varying effects on the pathogenicity, movement and survival of different EPN species (Kung *et al*., 1990; Shapiro-Ilan *et al*., 2006). Other abiotic factors that should be taken into consideration include UV radiation and pH (Shapiro-Ilan *et al*., 2006).

Biotic antagonists in the soil that rapidly reduce applied EPN numbers include a variety of organisms, such as bacteria, protozoans, phages, nematophagous fungi, predacious mites and other free living nematodes (Kaya, 2002).

**Environmental Safety and Entomopathogenic Nematode Use**

When investigating the potential use of biocontrol, whether or not such agents are completely ecologically safe requires consideration. Concerns include the potential effects that the biocontrol might

have on populations and species composition of non-target species (Bathon, 1996). In general, little is known about the effects of the introduction or augmentation of biological control agents on below-ground fauna (Somasekhar *et al*., 2002). Additionally, only a few studies have been done concerning the impact of EPNs on natural faunal communities (Bathon, 1996). Van Lenteren *et al*. (2003) have expressed concern that the current popularity of commercial inundative biological control might, in some cases, be conducted by people who are poorly trained in risk assessment, identification and evaluation of the biological agent. Despite concerns, adverse effects of applied EPNs seem unlikely to occur, as the population density decreases rapidly to background levels, followed by a patchy distribution of the applied EPNs due to the many acting biotic and abiotic antagonists (Bathon, 1996; Smits, 1996). Never the less, according to Gaugler (1988) and Ehlers (1996), EPNs have shown no mammalian pathogenicity. However, Bathon (1996) concludes, taking into consideration the findings of laboratory tests, that vertebrates cannot be included in the host range of EPNs, and that they are unaffected by the application of EPNs for pest control.

**Sustainable Agriculture**

The term ‘sustainable development’ in itself is contradictory and ambiguous. The concept of sustainability is popular among environmentalists, as it embraces an inclination towards the making of ecological and social changes, whereas development, at face value, is of primary concern to developers (Wackernagel & Rees, 1996; Dresner, 2002).

The World Commission on Environment and Development (WCED) in 1987 released the report known as *Our Common Future* (Hattingh, 2001), which included the now well-known definition of sustainable development as “development which meets the needs of the present without sacrificing the ability of future generations to meet their needs” (Dresner, 2002). The definition can easily be extended into the agricultural sector by simply replacing the word ‘development’ with the word ‘agriculture’.

Considering that agriculture occupies approximately 25-37% of the world’s land area (Avery, 1999), the adoption of new pest control methods, within our agricultural systems, that conserve and incorporate biodiversity might potentially benefit the natural environment and the agricultural sector. Recognising

such potential might be a step forward in shifting towards sustainable food production and biodiversity conservation. Sadly, there is little evidence of the global adoption of sustainable agricultural practices (Bowler, 2002). Chemical pesticides and fertilisers tend to diminish the soil's fertility and to harm many non-target biota (Lapkin, 1999). Development and cultivation has led to irreversible losses in terms of biodiversity, while habitat destruction has been, and still is, the leading cause of species extinction (Pimm & Raven, 2000).

With current extinction rates and levels of biodiversity and ecosystem service loss, a concerted effort should be made to change agricultural methods so as to contribute towards the alleviation of the global problem. The current rise of interest in, and increase in the number of investigations into, the use of EPNs within an IPM scheme is a positive move towards reducing chemical pesticide use in the pursuit of sustainable agriculture.

**Aims and Objectives**

The overall aim of the current study was to generate knowledge of, and awareness of how to control *P. ficus* in South African vineyards by using EPNs as a biological control agent.

The objectives of the study were:

- to determine which species and isolate of EPNs is the most effective in controlling *P. ficus* in the laboratory;
- to determine the compatibility of EPNs with agrochemicals used in the soil and the efficacy of controlling *P. ficus* that is found on the roots of grapevine.
- to determine the effects of environmental factors that may influence the movement and efficacy of EPNs as a biological control agent.

**The chapters of this study have been written as separate publishable papers, and, for this reason, some repetition in the different chapters has been unavoidable.**

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# CHAPTER 2

## The Potential use of South African Entomopathogenic Nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) to Control *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae)

Adapted from the paper published as: Le Vieux, P.D. & Malan, A.P., 2013. The Potential Use of Entomopathogenic Nematodes to Control *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae). *South African Journal of Enology and Viticulture* 34: 296-306.

**Abstract**

The vine mealybug, *Planococcus ficus* (Signoret), is considered to be the dominant mealybug species in South African vineyards (Kriegler, 1954; Walton, 2003). Laboratory bioassays were conducted to establish the potential of entomopathogenic nematodes (EPNs) as biocontrol agents of *P. ficus.* Six indigenous and two commercially available nematode species were screened for their efficacy in killing adult female *P. ficus*. The two indigenous species with the most promising results were *Heterorhabditis zealandica* and *Steinernema yirgalemense*, which were respectively responsible for 96% ± 2% and 65% ± 10% mortality. Tests were conducted to compare the efficacy of *H. bacteriophora* and *S. feltiae* produced *in vivo* and *in vitro*. *Heterorhabditis bacteriophora* showed no significant difference in efficacy between the two methods, albeit *in vivo* cultured *S. feltiae* produced a significantly higher mean mortality of 40% ± 4%, opposed to a 19% ± 4% mean mortality of *in vitro* produced infective juveniles (IJs). The capability of both *H. zealandica* and *S. yirgalemense* to complete their life cycles in the host and to produce a new cohort of IJs was demonstrated. Bioassays exposed the concentration-dependent susceptibility of *P. ficus* to *H. zealandica*, *S. yirgalemense* and commercially produced *H. bacteriophora* with $LC_{50}$ and $LC_{90}$ values of 19, 82; 13, 80; and 36, 555, respectively. Both *H. zealandica* and *S. yirgalemense* were able to move 15 cm vertically downward and infect *P. ficus*, having produced promising results, with respective mortalities of 82% ± 4.1% and 95% ± 1.4%. This study showed *P. ficus* to be a suitable host for *H. zealandica* and *S. yirgalemense*, with both nematode species showing considerable potential for future use in the field control of *P. ficus*.

## Introduction

Mealybugs (Pseudococcidae) are severe agricultural pests, which pose major problems for farmers (Miller *et al*., 2002). The vine mealybug, *Planococcus ficus* (Signoret), has shown to be the dominant mealybug species in South African vineyards (Kriegler, 1954; Walton, 2003), and is characteristically more economically damaging than any other mealybug species (Haviland *et al.*, 2005; Daane *et al*., 2008).

Compared to *Pseudococcus maritimus* (Ehrhorn), *Pseudococcus longispinus* (Targioni-Tozzetti) and *Pseudococcus viburni* (Signoret), *P. ficus* excrete more honeydew per individual, have both a faster development time and a higher reproductive rate of more than 250 eggs per female, and are able to feed on all parts of the vine throughout the year (Daane *et al*., 2003, 2008; Haviland *et al*., 2005). *Planococcus ficus* is a vector for Shiraz disease, corky bark disease and the grapevine leafroll associated virus 3 (GLRaV-3), making it a potential economic threat, even at low densities (Walton & Pringle, 2004b; Douglas & Kruger, 2008; Holm, 2008). Mealybugs are difficult to control with chemicals due to their cryptic lifestyles of hiding in crevices, under bark and below ground on roots where chemicals battle to reach (Walton & Pringle, 2004b). Their hydrophobic waxy secretions repel water-based insecticides, and they have the ability to develop resistance rapidly (Flaherty *et al*., 1982; Walton & Pringle, 2004b; Franco *et al*., 2009). There is, thus, a need for *P. ficus* to be controlled by means of new, effective methods.

Entomopathogenic nematodes (EPNs) of the Heterorhabditidae and Steinernematidae families occur in soils from most parts of the globe, and are deadly parasites to a wide range of insects (Stuart *et al*., 1997). Heterorhabditidae and Steinernematidae have a unique symbiotic association with the entomopathogenic bacteria genera *Photorhabdus* and *Xenorhabdus*, respectively, and together they effectively parasitise and kill their insect hosts (Ehlers, 2001). When encountering a suitable host, the free-living, non-feeding infective juvenile (IJ) enters the host via natural openings such as the anus, mouth or spiracles (Gaugler *et al*., 1997; Griffin *et al*., 2005). The bacteria grow rapidly within the nutrient-rich haemolymph and produce toxins and other metabolites that kill the host by means of inducing septicaemia within 24-48 hours of infection (Gaugler *et al*., 1997; Ehlers, 2001; Griffin *et al*., 2005).

An increasing amount of interest has been shown in the notable potential of Heterorhabditidae and Steinernematidae for inundative application as a biocontrol agent against economically important insect pests (Griffin *et al*., 2005). The advantages of using nematodes are that they actively seek out well-hidden hosts and combined with their associated bacteria, kill their hosts fast. Despite their large host range, they are not pathogenic to vertebrates. In addition, they are compatible with commercial rearing and application techniques (Gaugler, 1988; Bathon, 1996; Stuart *et al*., 1997). The commercial production of entomopathogenic nematodes as an environmentally safer option to the use of chemical insecticides can be regarded as being due to the increased understanding of their biology, improved production methods and storage formulations (Friedman, 1990; Ehlers, 1996). According to Le Vieux and Malan (2013), such increased understanding and commercial production of EPNs are both pertinent in the pursuit of sustainable agriculture, in helping to alleviate the global issues of species extinctions and ecosystem and biodiversity loss.

Unfortunately, only a few studies, as of yet, have been undertaken to test the efficacy of South African EPN species against insect pests. More so, fewer tests have been done that test the susceptibility of Pseudococcidae to EPNs. It is important that South African species of EPN should be discovered and tested. As a precautionary policy, strict regulations concerning the importing of exotic organisms into South Africa cover the importation of EPNs, among other such organisms (in terms of amended Act 18 of 1989, under the Agricultural Pest Act 36 of 1947) (Malan *et al*., 2011). Such regulations ultimately prevent the importing of exotic, commercially available EPNs prior to conducting a full impact study (Malan *et al*., 2006). The concern is that exotic nematode species might have effects on non-target South African organisms, while possibly displacing indigenous nematode species. In addition, exotic nematodes are not generally well adapted to local environmental conditions (Grewal *et al*., 2001; Ehlers, 2005). Increasing numbers of new South African EPN species with biocontrol potential are still in the process of being studied. Examples of such species concerned include *Steinernema citrae* Stokwe, Malan & Nguyen, 2010, *Heterorhabditis safricana* Malan, Nguyen & Tiedt, 2008, *Steinernema khoisanae* Nguyen, Malan & Gozel, 2006, *Heterorhabditis zealandica* Poinar, 1990, *Heterorhabditis bacteriophora* Poinar, 1976, *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt, 2013 and *Steinernema yirgalemense* Tesfamariam, Gozel, Gaugler & Adams, 2004 (Malan *et al*., 2006, 2011).

International studies of EPNs on mealybugs include the study that was conducted by Stuart *et al*. (1997), who tested the susceptibility of the *Dysmicoccus vaccinii* (Miller & Polavarapu) to four different nematode strains, using sand-dish bioassays. As a result of various tests, *H. bacteriophora* was found to be the most lethal EPN for use against *D. vaccinii.* Another test was undertaken by Alves *et al.* (2009), who tested various *Heterorhabditis* strains against the coffee root mealybug, *Dysmicoccus texensis* (Tinsley), proving that the majority of the species tested were successful at killing the host.

In a South African study by Stokwe (2009), who performed various bioassays on *Pseudococcus viburni* (Signoret), the obscure mealybug, *H. zealandica* was shown to be the most lethal candidate of 16 different local EPN strains. Both *H. zealandica* and *S. yirgalemense* were able to complete their life cycle and to reproduce in *P. viburni* (Stokwe & Malan, 2010). On the testing of host size susceptibility to *H. zealandica*, the adult and intermediate life stages were shown to be the most susceptible to EPN infection with 78% and 76% respectively (Stokwe & Malan, 2010). *Heterorhabditis zealandica* was found to be able to enter the core of *P. viburni* contaminated apples, enabling it to infect the mealybugs inside (Stokwe & Malan, 2010).

Another South African study was undertaken on mealybugs by Van Niekerk (2012), who conducted different bioassays and field trials to determine the potential of South African EPN isolates to control *P. citri*, the citrus mealybug. Tests showed that *P. citri* was most susceptible to *H. zealandica* (with 91% mortality) and *S. yirgalemense* (with 97% mortality) (Van Niekerk & Malan, 2012). Both species were able to complete their life cycles within the insect host, while *S. yirgalemense* proved to be more tolerant to lower levels of free water and faster at locating and infecting *P. citri* than *H. zealandica* was (Van Niekerk, 2012).

In the current study, the main objective was to establish the potential of EPNs to control adult female *P. ficus* under laboratory conditions. Screening was done to determine which nematode species was the most pathogenic to *P. ficus*. Once established, the best two candidates concerned were exposed to biological and life cycle studies within *P. ficus*. Further laboratory bioassays were conducted to establish ideal nematode concentrations; to compare virulence levels between commercially produced and

recycled commercially produced nematodes; and to establish the ability of nematodes to detect, move vertically and infect mealybugs in a sand column.

## Materials and Methods

### *Source of nematodes*

The six South African nematode species that were used in the current study were obtained from previous local surveys, and stored in the Stellenbosch University nematode collection (Table 2.1) (Malan *et al*., 2006, 2011). IJs of the six species were cultured *in vivo*, using the last instar of the greater wax moth larvae, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) at room temperature (Griffin *et al*., 2005). The IJ rearing and harvesting procedures were conducted according to the methods of Kaya and Stock (1997) and White (1927). IJs from the White trap were harvested within the first week of emergence and stored horizontally in 500-ml vented culture flasks containing approximately 150 ml distilled water at 14°C. The nematodes were used within a month after harvesting. To aid in aeration and nematode survival during storage, the culture flasks were shaken weekly. The two commercially produced and available nematode species were stored in the refrigerator for a few days before use. The nematode concentrations used for different experiments were calculated using the equation developed by Navon and Ascher (2000).

Table 2.1: South African *Steinernema* and *Heterorhabditis* species, isolate, habitat, locality and GenBank accession number used.

| **Species** | **Isolate** | **Habitat** | **Locality** | **GenBank accession number** |
|---|---|---|---|---|
| *S. khoisanae* | SF80 | Disturbed | Villiersdorp, Western Cape | DQ314289 |
| *S. citrae* | 141-C | Disturbed | Piketberg, Western Cape | EU740970 |
| *S. yirgalemense* | 157-C | Disturbed | Friedenheim, Mpumalanga | EU625295 |
| *H. bacteriophora* | SF351 | Disturbed | Wellington, Western Cape | FJ455843 |
| *H. safricana* | SF281 | Disturbed | Piketberg, Western Cape | EF488006 |
| *H. zealandica* | SF41 | Natural | Patensie, Eastern Cape | EU699436 |

(Malan *et al*., 2006, 2011)

*Source of insects*

To ensure a constant, reliable supply of healthy mealybugs for experiments, a laboratory culture must be established. Consequently, a colony *P. ficus* was reared at 25°C in the laboratory on fresh butternuts within a wooden, framed cage (650 mm × 350 mm × 590 mm). The front of the cage was transparent Perspex, for ease of viewing, with insect-proof mesh on the top and sides to allow for adequate ventilation. The bottom of the cage was covered with wax paper. Clean butternuts were placed against infested butternuts to allow for the quick and easy dispersal of the *P. ficus* individuals. To increase colony size, more butternuts were added, whereas rotting butternuts were discarded. Female mealybugs were removed from the butternuts using a fine paintbrush to minimise injury. The initial *P. ficus* colony was obtained from the Agricultural Research Council (ARC)-Infruitech-Nietvoorbij, Stellenbosch.

The *G. mellonella* larvae were reared on a diet consisting of a mixture of five parts baby cereal (Cerelac Nestlé™), brown bread flour, and bran, two parts yeast and wheatgerm, and one part honey, and kept in a growth chamber at 28°C.

*Bioassay protocol*

For the test arena 24-well bioassay trays (Flat bottom, Nunc™, Cat. No. 144530, Thermo Fisher Scientific (Pty) Ltd., Gauteng, Johannesburg, South Africa) were used. The bottom of each alternate well was lined with a circular (13-mm-diameter) piece of filter paper. Five trays were used for each treatment, as well as five trays for the control. A single adult female *P. ficus* was placed in each well and inoculated with the desired IJ concentration per 50 µl, using an Eppendorf micropipette. The *P. ficus* individuals in the controls received 50 µl of water only. To prevent the insects from escaping, each plate was covered with a glass pane inside the lid, and held closed with a rubber band. The trays were placed in plastic containers, lined with moistened paper towels, and closed with a lid, to ensure high humidity levels (RH ± 95%), in a dark growth chamber at 25 ± 2°C for 48 h. After two days, mortality was determined and dead individuals from the treatment were removed and rinsed of external IJs, using water, then placed in small Petri dishes, lined with moist filter paper, sealed with PARAFILM®, and placed back in the growth chamber at 25 ± 2°C for another 48 h. A water drop was then placed on each of the cadavers for dissection, and viewed with the aid of a stereomicroscope to validate that the deaths concerned had been

due to nematode infection. Cadavers with visible nematodes were recorded as having been infected, while others (in both the control and treatments) were recorded as having died from natural causes.

*Screening*

The 24-well bioassay protocol was used to test the ability of the six endemic EPN species (Table 2.1) and of two commercially produced EPN species to infect adult female *P. ficus* under optimal laboratory conditions. The bioassay protocol was followed, and in order to reduce any edge effects and so as to ensure an even distribution in the plate, the bottom of each alternative well was lined with a circular (13-mm-diameter) piece of filter paper. As a result of the carrying out of the above procedure, 12 wells were occupied per tray, each with a single mealybug. Five trays were used for the treatment of each nematode species, and five trays for the control. Each mealybug in the treatment was inoculated with 100 IJs / 50 µl of water. The procedure was repeated on a separate date, with a different batch of nematodes.

*Biological study*

The current qualitative study entailed detailing and recording the developmental progression of both *S. yirgalemense* and *H. zealandica* within adult female *P. ficus* individuals. Using the multiwell bioassay protocol, adult female mealybugs were infected, using 100 IJs of both nematode species. Two days post inoculation, 200 infected mealybugs of each nematode species were selected and rinsed with distilled water to remove excess IJs from the surface of the insect. A total of 50 insects was placed on four damp filter papers in 13-cm-diameter Petri dishes, and sealed with PARAFILM®. Twenty-five individuals for each nematode species were selected at random, dissected and inspected at a time, beginning at 48 h, and then at one to two days after that, until the life cycle was completed or seized to continue. The mean number of IJs that penetrated the mealybugs was recorded for the first two dissections, while the life cycle progression of the nematodes, and the colour changes in the mealybug, were recorded throughout. To establish whether the two nematode species would complete their life cycle in *P. ficus,* ten infected individuals were left in white traps, with the average IJ production per mealybug being calculated for both nematode species.

*Effect of nematode concentrations on levels of* P. ficus *mortality*

The effect of increasing concentrations of *S. yirgalemense, H. zealandica* and *H. bacteriophora* was tested. Five 24-well bioassay trays were used, of which eight evenly distributed wells were lined with a circular (13-mm-diameter) piece of filter paper, into which a single adult female mealybug was placed. The above-mentioned procedure was carried out for each of the nematode concentrations tested (0, 5, 10, 20, 40 and 80 IJs / 50 µl /mealybug). The trays were placed in plastic containers lined with moistened paper towels, and closed to ensure high humidity levels (RH ± 95%). They were then placed in a dark growth chamber at 25 ± 2°C for 48 h. The procedure was repeated on a separate date, using a new batch of nematodes.

*Virulence comparison between* in vivo *and* in vitro *nematodes*

The *in vitro,* commercially produced *S. feltiae* (CSf) and the commercially produced *H. bacteriophora* (CHb) were used to determine the ability of *in vitro* and *in vivo* cultured EPNs to infect *P. ficus*. The commercially produced nematodes were used to inoculate *G mellonella* larvae, and the emerged IJs were harvested as having been 'recycled' (*in vivo*) from white traps, and then used to inoculate the mealybugs. From this point on, they are, therefore, referred to in the current study as recycled commercially produced *S. feltiae* (RCSf) and recycled commercially produced *H. bacteriophora* (RCHb). The formulation IJs were also used to inoculate the mealybugs directly. The bioassay protocol was followed and 12 wells per tray were occupied, each with a single mealybug. Five trays were used for the treatment, as well as five trays for the control for each nematode species. Each mealybug in the treatment was inoculated with 100 IJs / 50 µl, with the procedure being repeated on a separate date.

*Vertical sand column test*

The ability of *S. yirgalemense* and *H. zealandica* to detect and infect adult female *P. ficus* in vertically placed sand columns was tested. Five mealybugs were placed in a single perforated (using a heated surgical needle) PCR 0.2 ml tube. The tube was then placed at the bottom of a 15 × 1.5 cm centrifuge tube. To sterilise the river sand used, it was frozen at -40°C, and dried in an oven overnight at 50°C. To ensure adequate moisture levels, a ratio of 1:10 v/v of water and sand was mixed. Each centrifuge tube was filled with sand to the top. A 13-mm-diameter piece of filter paper was inoculated with 500 IJs/ 50 µl.

The inoculated side was placed downward and left on top of the sand. The lid of the centrifuge tube was screwed on, and placed vertically in a dark growth chamber at 25 ± 2°C for 48 h. For each nematode species, 20 tubes (i.e. in total 100 mealybugs) were used, and the experiment was repeated on a separate date with a new batch of nematodes.

*Data analysis*

All statistical analyses were done using STATISTICA version 11 (StatSoft Inc. 2012). All data, except for the concentration trials, were corrected, in order to compensate for natural deaths, using Abbott's formula (Abbott, 1925). An analysis of variance (ANOVA) was used to analyse the data. A post-hoc comparison of means was done using Bonferroni's method, or, when residuals were not normally distributed, a bootstrap multi-comparison of means was conducted, with 95% confidence intervals (Efron & Tibshirani, 1993). Data from different test dates were pooled. A probit analysis was performed using Polo PC (LeOra Software 1987) to determine lethal nematode dosages (Finney, 1971).

**Results**

*Screening*

Data analysed with a two-way ANOVA showed no significant difference between the species and the two test dates ($F_{(7, 64)}$ = 1.189; $P$ = 0.32). Data from the two batches were pooled, showing significant differences ($F_{(7, 72)}$ = 26.263; $P$ < 0.001) when comparing the species. Percentage mortalities ranged from 5% ± 2% (*H. safricana*) to 96 ± 2% (*H. zealandica*) (Fig. 2.1). The 96% mortality caused by *H. zealandica* was significantly higher than the mortality that was obtained with all other isolates, except for *S. yirgalemense*, which had a 65% ± 10% mortality. The two commercially produced species, CSf and CHb, did not differ significantly from each other ($P$ = 0.23), although CSf was only responsible for a 19 ± 0.04% mortality, in comparison to CHb, with a 42% ± 7% mortality. Although the mean mortality differed by 23%, no significant difference was found between *S. yirgalemense* and commercially produced *H. bacteriophora* ($P$ = 0.23).

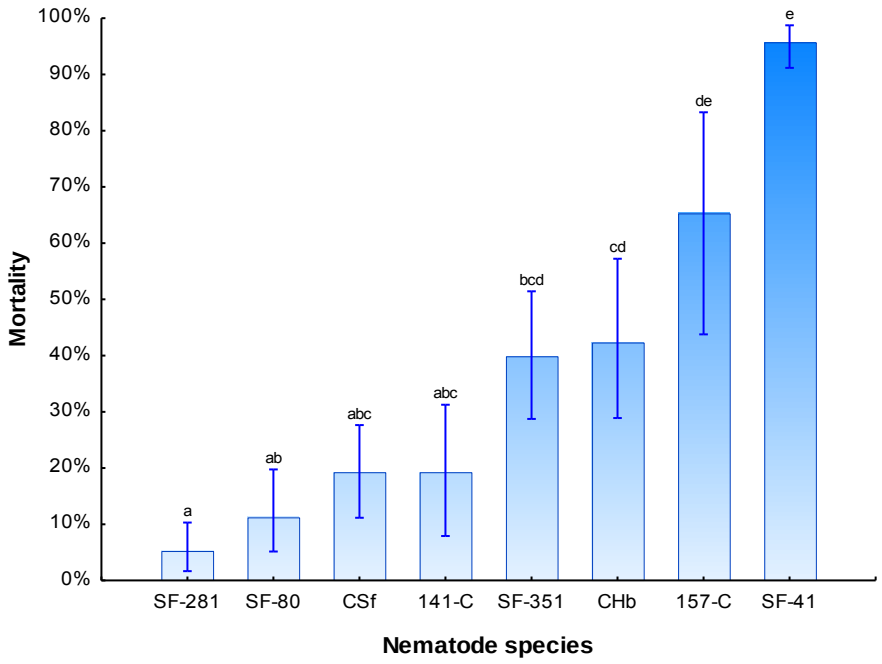


Fig. 2.1: The mean percentage mortality (95% confidence interval) of adult female *Planococcus ficus* inoculated with *Heterorhabditis safricana* (SF281), *Steinernema khoisanae* (SF80)*;* commercially produced *S. feltiae* (CSf)*; S. citrae* (141-C)*; H. bacteriophora* (SF351)*;* commercially produced *H. bacteriophora* (CHb)*; S. yirgalemense* (157-C) and *H. zealandica* (SF41), at a concentration of 100 IJs / 50 µl /insect after 48 h (one-way ANOVA; $F_{(7,72)} = 26.263$; $P < 0.001$). Bars sharing a common letter are not significantly different.

*Biological study*

For both *H. zealandica* and *S. yirgalemense,* their development and visual changes in the infected mealybugs were documented for a total of 14 days after inoculation (Table 2.2). *Planococcus ficus* individuals infected by *H. zealandica* ranged from yellow, orange to dark brown /black in colour within 48 h of infection (Fig. 2.2). When the yellow individuals were opened for inspection, their internal contents were easily separated in water in comparison to the internal contents of the orange and dark brown individuals, which were viscous and gummy in consistency. By day three, eggs were visible within the hermaphroditic *H. zealandica* adults (Fig. 2.3). On day six, larvae within the adults were apparent (Fig. 2.3), while the first emergent IJs were visible on day eight, completing the life cycle. A mixture of IJs and adults with larvae was present on day 10, but from day 14 onwards only IJs prevailed. The mean IJ

penetration was 13 (n = 50), with a penetration range of one to 37 IJs for a single mealybug. The mean IJ production per cadaver of *H. zealandica* was 300.

Mealybugs infected with *S. yirgalemense* also produced the same colour changes. Two days after inoculation, mealybug individuals were yellow, orange or brown/black in colour (Fig. 2.2). It was also observed that the darker the colour, the thicker the viscosity of the mealybugs' internal matrix was, due to the higher bacterial concentration levels. By day three, adults were present, with females containing eggs and males clearly being distinguishable, due to their diagnostic spicule (Fig. 2.4). On day six, larvae were present in the adult females. Day seven presented a mixture of larvae and adult females containing larvae. From day nine through to 14, IJs were present, while the pre-adults did not mature, and began dying by day 14. Some mealybug individuals did not contain IJs, but only pre-adults, which eventually died. The mean IJ penetration was six (n = 50), with a penetration range of one to 23 IJs for a single mealybug. The mean IJ production per cadaver for *S. yirgalemense* was 75.

Table 2.2: *Heterorhabditis zealandica* and *Steinernema yirgalemense* development in adult female *Planococcus ficus.*

| Nematode species | Number of days post inoculation | Developmental stage | Mean and range of IJ penetration (n = 50) |
|---|---|---|---|
| *H. zealandica* | 2 | Mixture of unrecovered IJs and recovered pre-adult stages | 13 (1-37) |
| | 3 | Mixture of recovered pre-adult stages and hermaphrodites with eggs | |
| | 4 | Hermaphrodites with eggs | - |
| | 6 | Mixture of hermaphrodites with eggs and hermaphrodites with larvae | - |
| | 8 | Mixture of hermaphrodites with eggs and larvae and first-generation IJs | - |
| | 9 | Mixture of hermaphrodites with eggs and larvae and IJs | - |
| | 10 | Mixture of hermaphrodites with larvae and IJs | - |
| | 14 | IJs only | - |
| *S. yirgalemense* | 2 | Mixture of unrecovered IJs and recovered pre-adult stages | 6 (1-23) |
| | 3 | Mixture of recovered pre-adults and adults (females containing eggs) | |
| | 4 | Mixture of pre-adults and adults (females containing eggs) | - |
| | 6 | Males and females filled with larvae | - |
| | 7 | Males and females filled with larvae and immature stages | - |
| | 9 | Immature stages and IJs | - |
| | 12 | Immature stages and IJs | - |
| | 14 | Immature stages and IJs | - |

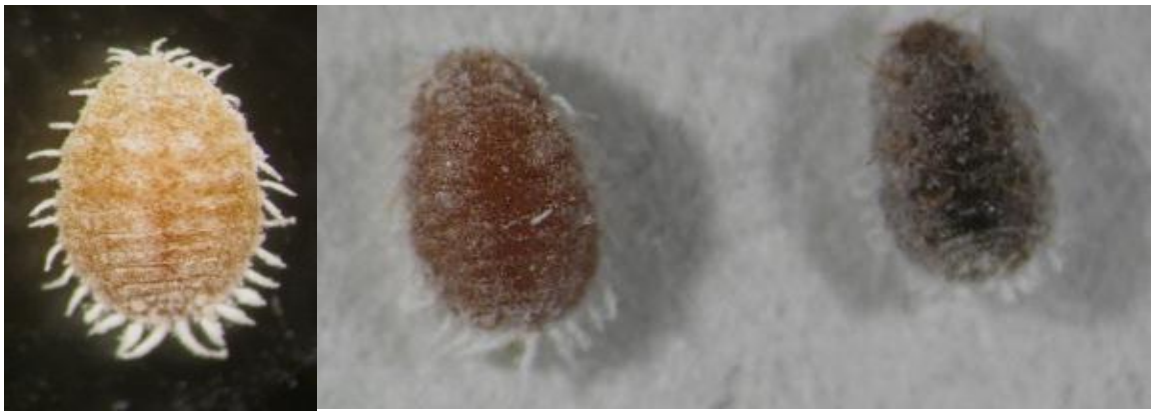

Fig. 2.2: Common colour differences of female *Planococcus ficus* 48 h after infection, with the control on the left, *Steinernema yirgalemense* (middle) and *Heterorhabditis zealandica* (right).

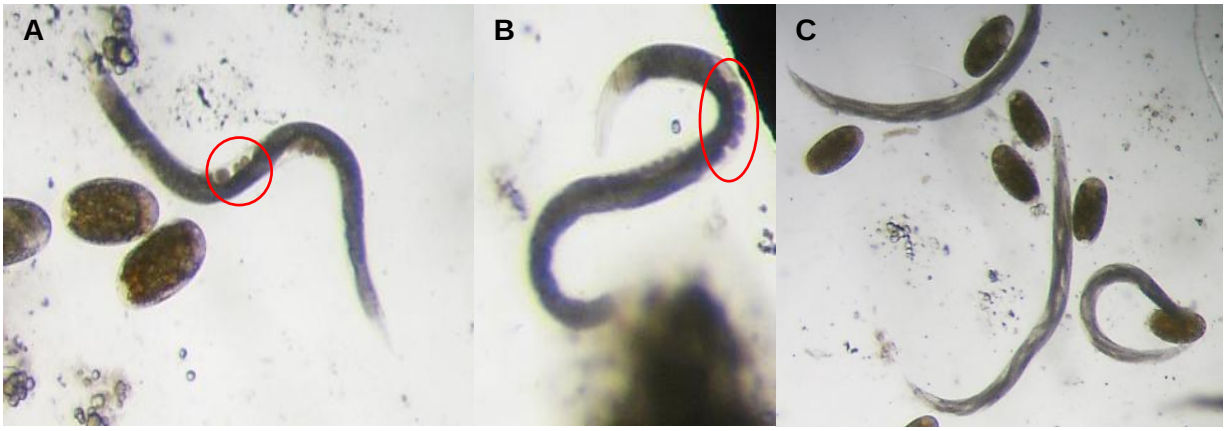


Fig. 2.3: After three days, large eggs are visibly present in hermaphroditic *Heterorhabditis zealandica* (A; B), with larvae being visible after 6 days (C).

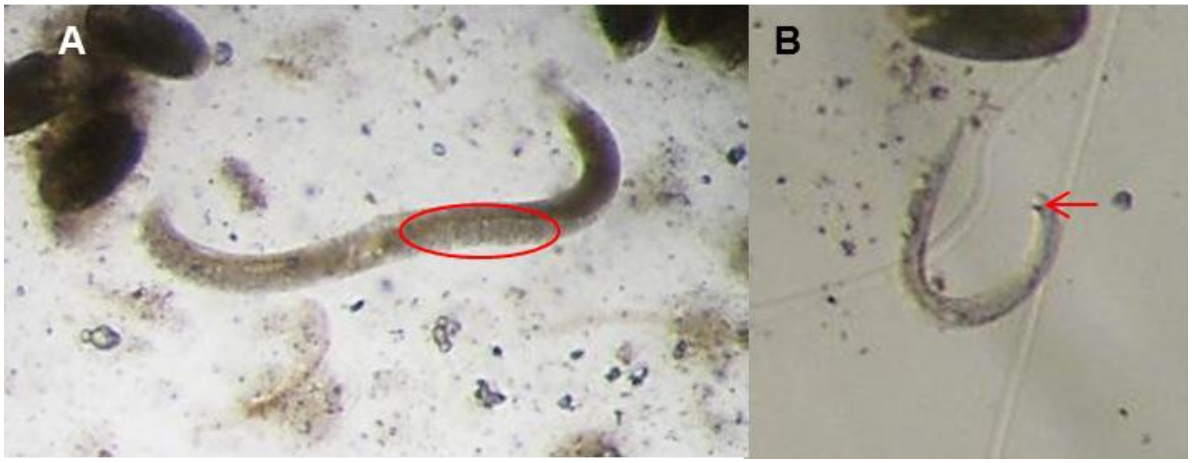


Fig. 2.4: After day three, eggs were present in female *Steinernema yirgalemense* (A), with while males being distinguishable by the presence of a spicule (B).

*Virulence comparison between nematodes grown* in vitro *and* in vivo

A four-way ANOVA showed no significant difference between batches (2 levels: batch 1 and batch 2, used on separate dates), between the species (2 levels: *S. feltiae* and *H. bacteriophora*), between formulations (recycled and not recycled) and between treatments (2 levels: treatment and control) ($F_{(1, 64)} = 0.05924$; $P = 0.808$).

Data from the two batches were pooled and a two-way ANOVA was used to compare species and formulations ($F_{(1, 36)} = 0.68787$; $P = 0.412$). Although no significant overall interaction was indicated, when investigating performances of the two species separately, there was a significant difference between RCSf and non-recycled CSf ($P = 0.048$). Percentage mortalities for RCHb and CHb were 54% ± 5% and 42% ± 5%, respectively, with RCHb being responsible for a 12% higher mortality in adult female *P. ficus* (Fig 2.5).

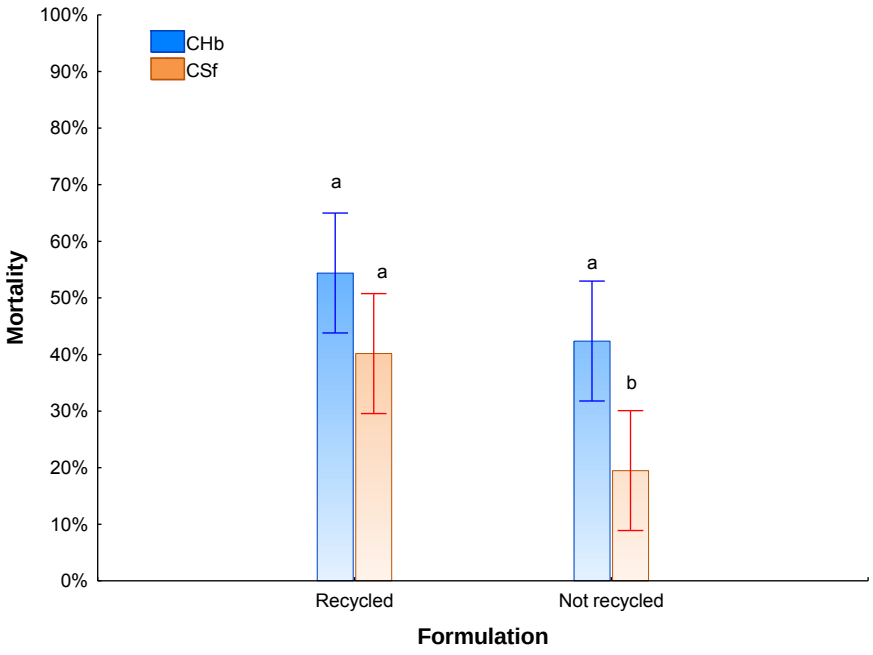


Fig. 2.5: The mean percentage mortality (95% confidence interval) of female adult *Planococcus ficus* infected with commercially produced *Heterorhabditis bacteriophora* and *Steinernema feltiae* (CHb and CSf) and recycled commercially produced *H. bacteriophora* and *S. feltiae* (RCHb and RCSf), at a concentration of 100 IJs / 50 µl /insect after 48 h (two-way ANOVA; ($F_{(1, 36)} = 0.68787$, $P = 0.412$). Bars sharing a common letter are not significantly different.

*Effect of nematode concentration on* Planococcus ficus *mortality*

Results analysed using a three-way ANOVA showed no significant difference between the species (3 levels: *S. yirgalemense*, *H. zealandica* and CHb), nematode concentration (6 levels: 0, 5, 10, 20, 40, 80 IJs /insect) and different batches (2 levels: batch 1 and batch 2, used on separate dates) ($F_{(10,\ 144)}$ = 1.549; $P$ = 0.13). Data from the two batches were pooled, and a two-way ANOVA comparing the different species and six concentrations showed significant differences ($F_{(10,\ 162)}$ = 2.828; $P$ < 0.05). The only significant difference at a given concentration was between the commercially produced *H. bacteriophora* (CHb) and the other two species, at a concentration of 80 IJs /mealybug ($P$ < 0.001). *Heterorhabditis bacteriophora* produced a 62% ± 7.4% mean mortality, while *H. zealandica* and *S. yirgalemense* both produced mean mortalities of 94% ± 2.7%. At all other given concentrations, there was no significant difference between the species (Fig. 2.6).

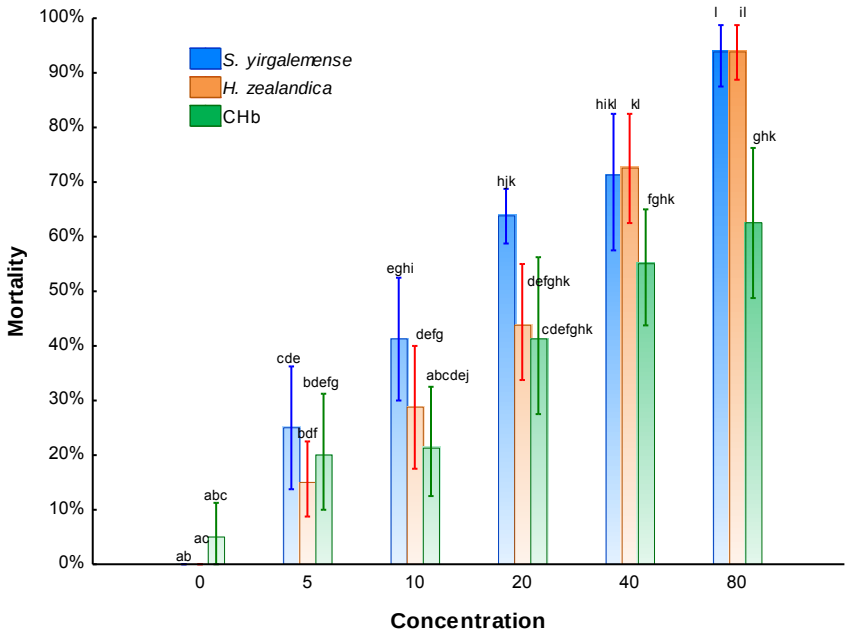


Fig. 2.6: The mean percentage mortality (95% confidence interval) of female adult *Planococcus ficus* infected with *Steinernema yirgalemense, Heterorhabditis bacteriophora* and commercially produced *Heterorhabditis bacteriophora* (CHb) at 0, 5, 10, 20, 40 and 80 IJs /mealybug after 48 h (two-way ANOVA; $F_{(10,\ 162)}$ = 2.828; $P$ < 0.05). Bars with different letters indicate a significant difference.

The $LC_{50}$ and $LC_{90}$ of *P. ficus*, after 24 h exposure to *H. zealandica, S. yirgalemense* and CHb, were 19 IJs /mealybug and 82 IJs /mealybug; 13 IJs /mealybug and 80 IJs /mealybug; 36 IJs /mealybug and 555 IJs /mealybug, respectively (Fig. 2.7). The $LC_{90}$ for the CHb extends beyond the scope of the graph.

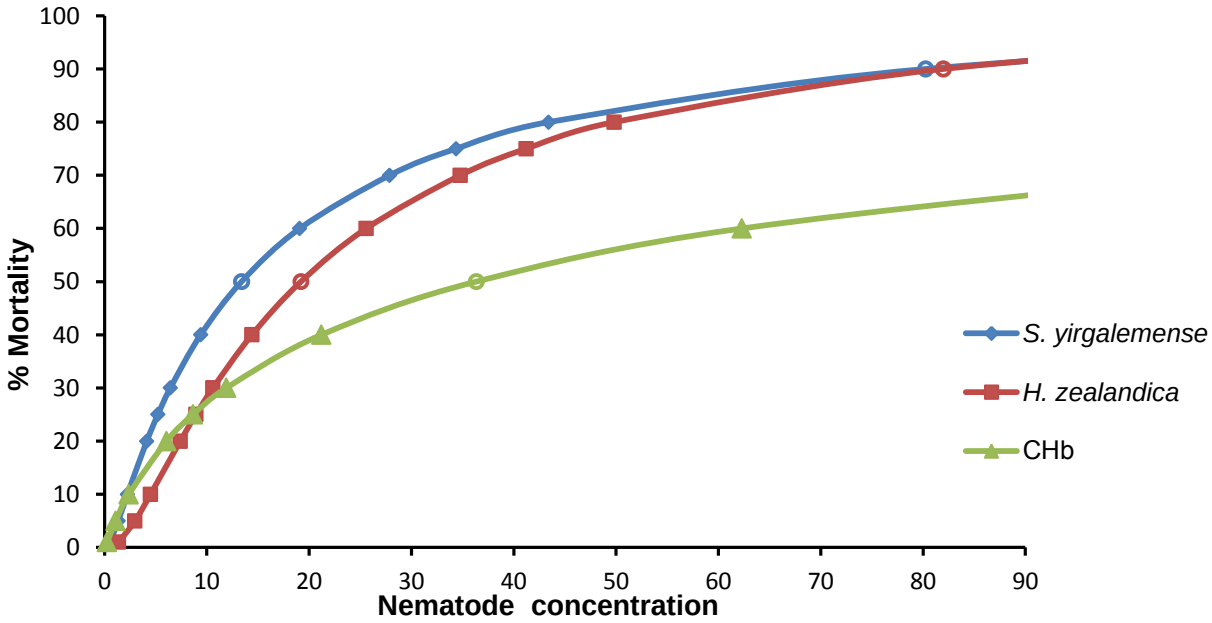


Fig 2.7: The mean percentage mortality of adult female *Planococcus ficus* 48 h after exposure to *Heterorhabditis zealandica, Steinernema yirgalemense* and commercially produced *Heterorhabditis bacteriophora* (CHb). The $LC_{50}$ and $LC_{90}$ values of each species are indicated on the curves by circular markers (probit analysis).

*Vertical sand column test*

The percentage mortality of the pooled data was analysed using a one-way ANOVA. A significant difference was found between the performance of *S. yirgalemense and H. zealandica* ($F_{(1, 78)} = 8.878$; $P = 0.003$). The mean percentage mortality for *H. zealandica* and *S. yirgalemense* was 82% ± 4.1% and 95% ± 1.4%, respectively (Fig. 2.8).

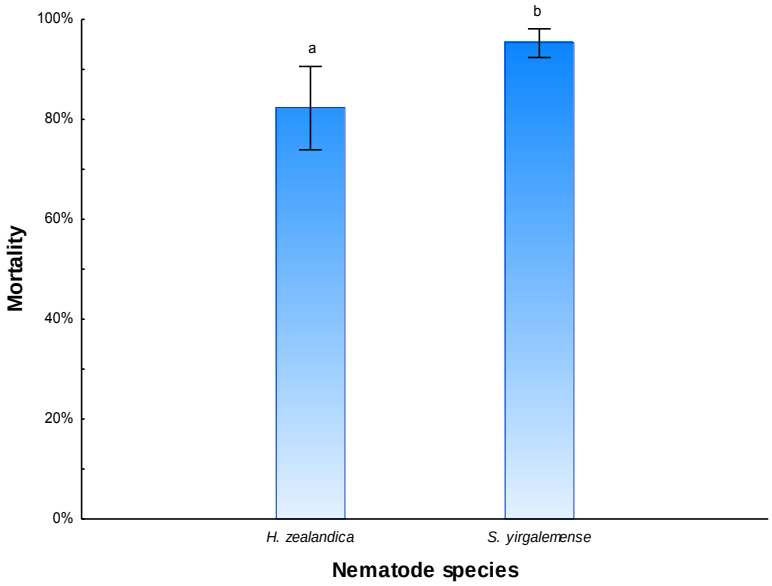


Fig. 2.8: The mean mortality (95% confidence interval) of female adult *Planococcus ficus* buried under 15 cm of sand after inoculation with *Heterorhabditis zealandica* and *Steinernema yirgalemense* at a concentration of 100 IJs /mealybug after 48 h (one-way ANOVA; $F_{(1, 78)} = 8.878$; $P = 0.003$). Different lettering indicates significant differences.

## Discussion

The study of the use of EPNs for the control of *P. ficus* is novel. In such investigations, initially determining the virulence of the different nematode species, under optimum environmental conditions, is the first and most important step when considering the potential use of EPNs as a biological control agent against *P. ficus.*

Research has shown, through the screening of six indigenous EPN species and two commercially produced species, that there are clear differences in pathogenicity between the nematode species concerned. Under optimal laboratory conditions, *H. zealandica* outperformed the other seven nematode species with a mean mortality of 96% ± 2%, while, in contrast, *H. safricana* scored a mean mortality of only 5% ± 2.6%. The results obtained illustrate the range in susceptibility of the host against the different species concerned. *Steinernema yirgalemense* produced the second best mean mortality result of 65% ± 10%, but with no significant difference for *H. zealandica* and *H. bacteriophora*. These results are similar

and complementary to earlier work that was conducted on different mealybug species, specifically on *P. viburni* by Stokwe (2009) and on *P. citri* by Van Niekerk and Malan (2012). *Heterorhabditis zealandica* and *S. yirgalemense* were within the top four most pathogenic species in the study conducted by Stokwe (2009), while they were the best two species, producing very high percentage mortalities of 91% and 97%, respectively, in Van Niekerk and Malan's (2012) study.

Interestingly, studies that were conducted on other insect pests have shown similar promising results concerning *H. zealandica* and *S. yirgalemense.* Specifically, *H. zealandica* was selected as the best candidate for the control of codling moth, *Cydia pomonella* (L.), by De Waal (2008), and Malan *et al*. (2011) showed *S*. *yirgalemense* to be highly virulent against false codling moth, *Thaumatotibia leucotreta* (Meyrick), while the work that was conducted by Ferreira and Malan (2013) held *H. zealandica* responsible for the highest mortality levels of the banded fruit weevil, *Phlyctinus callosus* (Schönherr). Of the commercially produced EPNs, *H. bacteriophora* was found not to be significantly different from commercially produced *S. feltiae.* General performances between the two families are varied, and, considering that *H. zealandica* and *S. yirgalemense* were the best, potential arguments concerning the general superior performance of heterorhabditids in relation to steinernematids (leading to speculation on heterorhabditids and the possession of a dorsal tooth that might promote increased levels of penetration, thus encouraging better performance) can be disregarded. When considering the current study and previous studies, these two species clearly displayed highly virulent qualities to a variety of different insect pests, including *P. ficus*, and were, thus, selected for further tests.

A biological study was done to gain insight into whether or not the EPNs of interest can develop and complete their life cycle in *P. ficus*. If they are able to do so it will affect the success and persistence of the biocontrol agent in vineyards, where it could have significant effects on not only mealybugs, but also on other soil stages of pest insects such as *P. callosus*.

When inspecting the cadavers two days after inoculation, the mean penetration number of *H. zealandica* exceeded the number of *S. yirgalemense* with a mean penetration number of 16 and 6, respectively. The results obtained were complemented by the mean penetration numbers established by Stokwe (2009) and Van Niekerk and Malan (2012) whom both had higher penetration levels with *H.*

*zealandica* in comparison to *S. yirgalemense*. The higher penetration number of *H. zealandica* corresponds to its superior performance in the screening test. There is an apparent direct relationship between penetration and insecticidal activity, which is supported by Hominick and Reid (1990), who assume that the nematode with the highest efficacy against the insect of interest would also have the best invasion efficacy. The findings of various other studies that have been conducted on different insect pests are in accordance with such findings (Kondo & Ishibashi, 1986; Mannion & Jansson, 1993; Shannag *et al*., 1994; Garcia Del Pino & Morton, 2005). The relatively low penetration numbers that have been obtained in comparison to the numbers that have been obtained with other insect hosts can be ascribed to the small size of the adult female *P. ficus*, which is approximately 4 mm in length and 2 mm in width.

Although both nematode species completed their life cycles, the amount of time that was taken to do so vary by a day or two in comparison to the amount of time that was taken in biological studies conducted by Stokwe (2009) and by Van Niekerk and Malan (2012). The variation could be attributed to the differing sizes of the mealybugs concerned, although they were very similar. The life cycle of *S. yirgalemense* was found to be longer in *P. ficus,* opposed to shorter, as had been found in the other two studies, and with the associated mealybug species. The development of *S. yirgalemense* in *P. ficus* was similar to that which is described in the findings of Van Niekerk and Malan (2012) in relation to *P. citri*, which was found to produce IJs, although, in some instances, the nematodes only developed to a certain stage, and then perished. In general, the factors that cause the development of IJs and movement out of the host are the availability of food and the overcrowding of nematodes in the host cadaver (Ehlers, 2001; Adams & Nguyen, 2002; Griffin *et al*, 2005). Thus, overcrowding and/or nutrient deficiencies will prompt the development of IJs and their movement out of the host in search of a new host. According to Smart (1995), the time taken from IJ entry into the host, until IJ emergence, is also dependent on the nematode species and on the ambient temperatures concerned. Such factors could explain the differences in the development and in the developmental times between the two EPN species within the three different mealybugs involved.

According to Gaugler and Georgis (1991), the quality of inoculum produced by various methods has received little attention. Accordingly, two EPN species that had been commercially produced in a liquid

medium and stored in a unique formula were tested against the same commercially produced species that were recycled through wax moth larvae. In both instances, the recycled, *in vivo* individuals had a higher mean mortality. For *H. bacteriophora*, the recycled IJs caused a mean mortality of 54% ± 5%, as opposed to 42% ± 5%, with no significant difference in effect to the results that were obtained with the commercially produced individuals. A significant difference was, however, detected between the commercially produced and recycled IJs of *S. feltiae*, with respective mean mortalities of 19% ± 13% and 40% ± 14%.

Due to the fact that *in vivo* production of EPNs is laborious, and limited by scale and economy, it is important that commercially produced nematodes are able to reach their full potential as biopesticides (Gaugler & Georgis, 1991; Ehlers, 2001). Unfortunately, many disadvantages are associated with large-scale production (Ehlers, 2001). Besides the problems that are associated with the actual mass (*in vitro*) culturing process itself, transport and storage, some studies have shown the pathogenicity of the EPNs to have also been compromised during the *in vitro* process. For example Yang *et al*. (1997) found that the quality of EPNs is influenced by the type of culture medium concerned. Both Gaugler and Georgis (1991) and Yang *et al*. (1997) found *in vivo* culturing methods to produce IJs with higher fatty acid content. With a reduction in the fatty acid content of IJs, it seems that there is a general reduction in pathogenicity (Gaugler & Georgis, 1991). In comparison it has been found that there is no difference in the total fatty acid content of IJs when produced *in vivo* (R-U.Ehlers, personal communication, 2013). The most likely reason for a reduction in pathogenicity includes prolonged storage and suboptimal storage conditions (Gaugler & Georgis, 1991).

A definite positive relationship exists between the concentration of all three nematode species used for inoculation and the percentage mortality of *P. ficus*. For all three species, there was no significant difference between the percentage mortalities at a given concentration besides that of 80 IJs /mealybug. No significant differences were detected between *H. zealandica* and *S. yirgalemense* and commercially produced *H. bacteriophora* from the inoculum concentration of 10 IJs /mealybug upward. The $LC_{50}$ and $LC_{90}$ values for *H. zealandica* in the current study were 19 and 82 IJs respectively, with the values concerned being very similar to the $LC_{50}$ and $LC_{90}$ values for *S. yirgalemense* at 13 and 80, respectively.

In contrast, the $LC_{50}$ and $LC_{90}$ for CHb were greater than they were for both *H. zealandica* and *S. yirgalemense*, with values of 36 and 555, respectively. The similarity in percentage *P. ficus* mortality caused by *H. zealandica* and *S. yirgalemense* in the concentration test was common to the *P. citrus* percentage mortalities found by Van Niekerk and Malan (2012). Despite such a finding, the $LC_{50}$ value of *H. zealandica* in the current study was similar to the $LC_{50}$ at 11 in Van Niekerk and Malan's (2012) study, although the $LC_{90}$ values differed greatly, with Van Niekerk and Malan (2012) obtaining a value of 162 nematodes, as opposed to 82, which is nearly double the amount. From the findings that were made in the present study, as well as in previous studies, *P. ficus* and *P. citri* have clearly been shown to be more susceptible to *H. zealandica* than is *P. viburni*. Stokwe (2009), in contrast, attained $LC_{50}$ and $LC_{90}$ values of 54 and 330 for *P. viburni*. The $LD_{90}$ values of *H. zealandica* and *S. yirgalemense*, in the current study, give a fair indication of nematode concentrations to be used in future field studies and trials.

Sand column tests were conducted to predict the potential performance of the two best EPN candidates in the field, while establishing which nematode species would be used for future field trials. Field soil applications are being considered, based on the fact that *P. ficus* has been found to move down to the lower regions, and underground onto the roots of the vine to overwinter, providing an opportunity to control them effectively with EPNs (Walton & Pringle, 2004a). Of the two nematode species, *S. yirgalemense* outperformed *H. zealandica* significantly, with mean mortalities of 95% ± 1.4% and 82% ± 4.1%, respectively. Despite such an outcome, both candidates performed well, and *H. zealandica* should not be disregarded completely, especially when considering previous works that have been performed and the successful results that have already been achieved. Ferreira and Malan (2013) conducted soil column tests by inoculating *P. callosus* larvae placed at different depths, with the deepest at 15 cm, with *H. zealandica*, which were then left for seven days. No significant differences in mortality were found between the depths of burial concerned. In the current study, despite nematodes being given only two days to detect and to infect *P. ficus* placed 15 cm deep, high mortality levels were, nevertheless, obtained. The reason for the better performance of *S. yirgalemense* is unclear, as there are many factors which could be at play. For instance, different soil types have been found by Koppenhoffer and Fuzy (2006) to influence the infectivity of different nematode species. To place an onus on their potentially different foraging strategies would be weak reasoning, when considering that a single nematode species

can be placed on a continuum ranging from cruise to ambush, while single nematode species have been found to shift along such a continuum (Lewis *et al*., 1992, 2006). Ultimately, the vertical movement of both nematode species tested in the current study were adequate in locating and infecting *P. ficus* below the soil to a depth of at least 15 cm.

According to results from the various bioassays, both *S. yirgalemense* and *H. zealandica* have displayed potential as good candidates for the control of *P. ficus*. This suggests the need for further studies to be conducted, in the hope of developing an adequate infrastructure for the commercial production of South African EPNs. The commercial production and the use of indigenous nematode species, as opposed to exotic species, for the control of agricultural pests such as *P. ficus* should avoid having to deal with potential environmental issues in the foreseeable future. Both *S. yirgalemense* and *H. zealandica* performed similarly throughout the bioassays, with *S. yirgalemense* generally doing slightly better, indicating the potential for better infield performance, where it will be likely to experience suboptimal conditions. *Steinernema yirgalemense* performed well in the soil column tests, which offers possibilities when considering the vertical movement of *P. ficus* down the trunk of the vine and underground onto the roots in late autumn and winter. As a result, the negative aspects of foliar application can possibly be avoided when using EPNs in an IPM scheme to control *P. ficus*, as soil and lower-trunk application is possible. During the winter months, moisture levels are generally relatively high and the temperatures are relatively cool. Further tests should be conducted in the field to confirm such speculations, and to answer queries of when and how to apply the nematode, as well as in which concentrations. In addition, their compatibility with agrochemicals should be determined.

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# CHAPTER 3

## Efficacy and Persistence of Entomopathogenic Nematodes for the Control of Soil-inhabiting Mealybugs (*Planococcus ficus*) in Vineyards


### Abstract

In South Africa the most common method of mealybug control has been the use of chemical insecticides, such as organophosphates. Alternatively, entomopathogenic nematodes (EPNs) of the families Heterorhabditidae and Steinernematidae are lethal parasites of a range of soil-dwelling insects. They can potentially be used within an integrated pest management (IPM) programme to control *Planococcus ficus*, which has been found to occur on grapevine roots. *Steinernema yirgalemense* was applied to the soil of two vineyards with *P. ficus*, contained in pierced Eppendorf tubes, buried 15 cm into the soil, and mortalities of up to 52% after 48 h were obtained. For the Welgevallen vineyard, *S. yirgalemense* were steadily persisting at three months post application. Studies concerning the simultaneous use of EPNs with the agrochemical imidacloprid showed that neither the viability nor the virulence of the EPNs was compromised. Tests were conducted to establish the possible production of scavenger deterrent factors by *H. zealandica* and *S. yirgalemense*. Of the cadavers that were presented 6 days after nematode infection, 49% of the *H. zealandica* and 60% of the *S. yirgalemense* infected cadavers were left intact. These studies showed that EPNs, and specifically *S. yirgalemense*, have promising potential to be used as a biological control agent for the control of soil populations of *P. ficus* alongside the use of imidacloprid within an IPM programme.


### Introduction

*Planococcus ficus* (Signoret) is currently regarded as both the dominant mealybug species, and a major pest insect of the South African table grape and wine industries (Kriegler, 1954; Walton, 2003). It not only causes cosmetic damage to grape bunches, but lowers grape production by transmitting various plant diseases and viruses, resulting in it being characteristically more economically damaging than any other mealybug species (Greiger *et al.*, 2001; Haviland *et al.*, 2005; Daane *et al*., 2008; Holm, 2008).

Up to date the most common method of mealybug control in South Africa has been the use of such chemical insecticides as organophosphates (Walton *et al*., 2004; Daane *et al*., 2006; Holm, 2008). Mealybugs are difficult to control using chemicals as they hide deep in crevices, under bark and on the roots where chemicals battle to reach (Walton & Pringle, 2004b). Another problem concerning the use of chemical pesticides is the ability of mealybugs to rapidly build up resistance (Flaherty *et al*., 1982; Walton & Pringle, 2004b; Franco *et al*., 2009). An alternative to primarily using chemicals in controlling *P. ficus* is to use entomopathogenic nematodes (EPNs) within an integrated pest management (IPM) scheme.

EPNs of the families Heterorhabditidae and Steinernematidae are found naturally in soils, where they are lethal parasites of a wide range of insects with soil-dwelling life stages (Foltan & Puza, 2009; Gulcu *et al*., 2012). Heterorhabditids and steinernematids both have obligatory symbiotic associations with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Boemare, 2001; Ehlers, 2001; Griffin *et al*., 2005). Both families have a life stage that is free-living and non-feeding, known as an infective juvenile (IJ), which is well adapted to long-term survival in the soil, where it seeks out an insect host (Ehlers, 2001). Once the IJ locates a suitable host, it enters the host body via natural openings, moves through the thin lining of the intestine, releasing its associated bacteria in the haemocoel, and killing the host within 24-48 h (Gulcu *et al*., 2012). Some species are currently commercially produced and used as effective biological control agents (Kaya & Gaugler, 1993 Hazir *et al*., 2003).

Symbiotic relationships within the agro-ecosystem need to be acknowledged, which may hinder or influence the process of controlling the vine mealybug. Mealybugs and ants are known to have a mutualistic symbiosis, whereby the ants obtain carbohydrate-rich honeydew from the mealybug, while in return providing it with protection, sanitation and transport (Mgocheki & Addison, 2009). Thus, ants can potentially increase mealybug pest problems by interfering with augmentative and natural biological control, and through assisting their dispersal (Phillips & Sherk, 1991; Daane *et al*., 2008). The most common pest ant species in South African vineyards include *Linepithema humile* (Mayr) (Argentine ant), *Anoplolepis steingroeveri* (Forel) (black pugnacious ant) and *Anoplolepis custodiens* (Smith) (common pugnacious ant) (Addison, 2002).

When using EPNs in an IPM scheme, it is important to determine the compatibility and interactions of the EPNs with agrochemicals such as pesticides (García del Pino & Jové, 2005; Gutiérrez *et al*., 2008). A previous South African study by Van Niekerk (2012) investigated the compatibility of two endemic nematodes *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2004 and *Heterorhabditis zealandica* Poinar 1990 with two biopesticides,Helicovir™ (nucleopolyhedro virus) and Cryptogran™ (*Cryptophlebia leucotreta* granulovirus), two adjuvants (Nu-Film-P® and Zeba®), and one insecticide (Cyperphos 500 E.C.®). They found no significant reduction in the levels of IJ infectivity in either of the nematode species when they were exposed to any of the formulated products.

The use of EPNs is generally more suited to controlling the soil stages of insect life cycles and soil-dwelling insect pests (Wilson & Gaugler, 2004). This is encouraging to know, in the light of the fact that the vine mealybug has a distinctive vertical seasonal movement on grapevines. Although the greater part of the population is found above ground, they have also been found to occur on vine roots down to a depth of 30 cm (Walton & Pringle, 2004a; De Villiers, 2006).

Nematode infectivity and persistence in the soil are both important factors when they are used as a biocontrol agent, both which are affected by many abiotic, biotic and application factors. A variety of abiotic factors, including soil moisture and textures, temperature and relative humidity have differing effects on the pathogenicity, movement and persistence of different nematode species (Kung *et al*., 1990, 1991; Shapiro-Ilan *et al*., 2006). Biotic adversaries in the soil that reduce applied EPN numbers include a variety of organisms, such as protozoans, predacious mites, bacteria, nematophagous fungi, and other free-living and competitor nematodes (Kaya, 2002; Campos-Herrera *et al*., 2012; Duncan *et al*., 2007).

The main objectives of the current study were to establish the efficiency of controlling soil populations of *P. ficus* using EPNs, while determining the in-field soil persistence of the applied nematodes. Considering the use of EPNs in an IPM scheme, the compatibility of simultaneously using EPNs with the pesticide imidacloprid (Confidor®), was determined. Acknowledging the mutualistic symbiotic relationship existing between ants and mealybugs, investigations concerning their potential interference with the use of EPNs as an inundative biological control agent for *P. ficus* was also investigated.

## Materials and Methods

*Source of nematodes and insects*

The IJ rearing and harvesting procedures were carried out according to the methods of Kaya and Stock (1997) using codling moth, *Cydia pomonella* (Linnaeus) (Lepidotera: Tortricidae), larvae at room temperature. IJs from the White trap (White, 1927) were harvested within the first week of emergence, and stored horizontally in 500-ml vented culture flasks containing approximately 150 ml of distilled water at 14°C. The nematodes were used within a month after harvesting. To aid in aeration and nematode survival during storage, the culture flasks were shaken weekly. The two local EPN species used, *H. zealandica* (SF 41) and *S. yirgalemense* (157-C), were originally obtained from previous local surveys, which were stored in the Stellenbosch University nematode collection (Malan *et al.*, 2006, 2011). The two commercially available nematode species *Steinernema feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976 were obtained from e-nema (Raisdorf, Germany) and stored in the fridge at 4°C before use.

A laboratory colony of *P. ficus* was established and reared at 25°C on butternuts. Initially, individuals were obtained from the Agricultural Research Council (ARC)-Infruitech-Nietvoorbij, Stellenbosch. *Cydia pomonella* eggs and artificial diet were obtained from obtained from Entomon Technologies (Pty) (Ltd), Stellenbosch, reared to last-instar larvae under diapausing conditions [photoperiod 10:14 (L:D)], at 25˚C and 60% humidity and stored in the diet in a closed container in a cold room at 5˚C until needed.

*Baseline sampling and soil properties*

Soil samples from each treatment vine (from both study sights, Welgevallen and Nietvoorbij (ARC)-Infruitech-Nietvoorbij, in Stellenbosch) were collected in 350-ml plastic containers. Prior to nematode application, ten mealworms, *Tenebrio molitor* (Linnaeus), were placed in the container and left for 7 days. Dead individuals were removed and placed on moist filter paper in a Petri dish, sealed with PARAFILM®, and left to incubate at 25˚C for 24 h, then placed on White traps to identify local EPN populations and species, to establish a baseline for the field trials.

Soil samples were collected from both study sites and analysed by Bemlab, a SANAS-accredited testing laboratory. A mechanical-3-fraction analysis, to determine the soil classification as well as the percentage of sand, clay, silt and the pH and the electrical conductivity (EC) of the two sites was determined.

*Effects of imidacloprid on survival and virulence*

The survival of *S. feltiae* and *H. bacteriophora* with the insecticide imidacloprid (Confidor®) was tested. The pesticide was prepared at twice the recommended dose. Suspensions of both nematode species were prepared at a concentration of 4000 IJs/ml. Treatments contained a mixture of 1 ml of nematode suspension in water and 1 ml of the pesticide solution, which was added to a Petri dish, sealed with PARAFILM® and left in a growth chamber at 25 ± 2ºC. Controls contained 1 ml of nematode suspension and 1 ml of water. Five treatment and five control Petri dishes were prepared for both nematode species. Nematode survival was estimated by means of repeatedly collecting 10 µl samples from each Petri dish, until 50 IJs were counted, of which individuals were recorded as being either dead or alive. Samples were taken directly after preparation (0 h), and then again after 6, 12, and 24 h.

To establish the virulence of both nematode species post exposure to the insecticide, 5 ml at 4000 IJs/ml of each treatment was prepared and kept in a growth chamber for 24 h at 25 ± 2ºC. After 24 h the 5 ml nematode/pesticide solution was diluted in 1 L of distilled water, in a measuring cylinder. After the nematodes had been allowed to settle to the bottom, excess liquid was then siphoned off, leaving 10 ml behind. The remaining 10 ml was used to inoculate five Petri dishes, containing ten codling moth larvae each, at a concentration of 100 IJs/insect. Five control Petri dishes, containing 10 codling moth larvae each, received water only, and both were left for 48 h in a growth chamber at 25 ± 2ºC. The codling moth larvae were then assessed for infection. The experiment for both nematode species was repeated on a different date.

*In-field soil application and infectivity of* Steinernema yirgalemense

Two study sites, Welgevallen and Nietvoorbij (ARC)-Infruitech-Nietvoorbij, in Stellenbosch were selected to conduct field trials. Welgevallen is situated at 33˚ 56' 28" S; 18˚ 51' 46" E, at an elevation of 117

meters in the foothills of the surrounding mountains, while Nietvoorbij is situated at 33˚ 54' 27" S; 18˚ 52' 12" E at an elevation of 236 meters, facing in a south-east direction. For both sites, a randomised design was performed using four treatments with concentrations of 0, 20, 40 and 80 IJs/cm$^2$. There were eight vines per treatment (*n* = 32 vines), with six rows of vines, of which the first and last vine, and the first and the last row, were left untreated to avoid any possible edge effects. Eight vines from each row were selected, ensuring that every second vine was left untreated to prevent cross-contamination of treatments. Two perforated (using a heated surgical needle) 0.2-ml Eppendorf tubes were tied together with cotton thread. After placing 5 adult female mealybugs in each tube, the respective lids were closed, and the tubes were buried 15 cm deep in the soil, as close to each treatment vine trunk as possible. The thread was left extending above the ground to enable easy detection and withdrawal. An area of 80 × 100 cm was measured around each treatment vine (Fig. 3.1).

The desired number of S. *yirgalemense* for each treatment was prepared in 200 ml of water and sprayed as evenly as possible onto the soil using a handheld spray bottle. Once the mealybugs in the tubes and four i-buttons were buried, each treatment vine was watered with 10 L of water, and the nematode solutions applied. One day later they were watered again (if there was no natural rainfall or irrigation), and after 48 h the mealybugs were removed from the soil. The mealybugs were rinsed with water, placed in small Petri dishes lined with moist filter paper, sealed with PARAFILM®, and placed in a growth chamber at 25 ± 2°C, where they were left to incubate at 25˚C for 24 h. The mealybugs were then dissected to confirm infection.


Fig. 3.1: A marked treatment vine with a measured area of 80 × 100 cm for EPN application.

*In-field soil persistence of* Steinernema yirgalemense

The same sites at Welgevallen and Nietvoorbij were used to conduct persistence trials. Instead of using *P. ficus,* codling moth larvae, with a high susceptibility to *S. yirgalemense* (De Waal *et al*., 2011), were used. Five perforated 0.2 ml Eppendorf tubes were tied together with cotton thread, with a single larva being placed in each tube. The tubes were buried as close as possible to the treatment vine trunks, with the thread extending above the soil. Larvae were left for five days in the soil, watered on the first day with 10 L of water, and then retrieved and placed on moist filter paper in a Petri dish. The dish was sealed with PARAFILM® and left to incubate for 24 h at 25˚C. After which, the individuals were dissected to confirm infection. This process was repeated in time intervals of one week, two weeks, a month, and three months after the date of EPN application. For each date, four i-buttons were buried to record soil temperatures.

*Ant deterrent factors of* Heterorhabditis zealandica *and* Steinernema yirgalemense

Ten *C. pomonella* larvae were placed in five 13-cm Petri dishes, and lined with moist filter paper. Petri dishes were inoculated with 100 IJs/larvae (1000 IJs in total), sealed with PARAFILM®, and left in a dark growth chamber at 25 ± 2°C for four and six days. This was repeated for both *S. yirgalemense* and *H.*

*zealandica*, while control larvae were freeze killed. Two shaded Argentine ant *Linepithema humile* (Mayr) nests were identified and used as study sites. At each nest, 25 codling moth larvae cadavers infected with *H. zealandica*, 25 infected with *S. yirgalemense* and 25 freeze-killed larvae (control) were individually placed in single perforated PCR 0.2-ml tubes. They were all linked (alternating between cadaver types, orange caps containing *S. yirgalemense*, black caps containing *H. zealandica* and clear caps containing controls) together by a thread (Fig. 3.2) and left for 24 hours. The procedure was repeated on a different date, with a single i-button used at each nest, for the duration of the experiment. The cadavers were taken back to the laboratory, where they were categorised into 'intact', 'bitten', and 'consumed'. The tubes and the tiny holes burnt in them ensured that only ants could reach the cadavers.

Fig. 3.2: Alternating *Heterorhabditis zealandica*, *Steinernema yirgalemense* and freeze-killed *Cydia pomonella* larvae cadavers, placed in perforated Eppendorph tubes and connected by thread.

*Data analysis*

All statistical analyses were done using STATISTICA version 11 (StatSoft Inc. 2012). An analysis of variance (ANOVA) was used to analyse the data. A post-hoc comparison of means was used employing Bonferroni's method, or, when residuals were not normally distributed, a bootstrap multi-comparison of means was conducted, with 95% confidence intervals (Efron & Tibshirani, 1993). Data from different test dates were pooled if no significant differences were found between the date and treatment.

## Results

*Baseline sampling and soil properties*

At Welgevallen 6.25%, and at Nietvoorbij 9.69%, of the vines used in the study were naturally infected with *H. bacteriophora*. The bacteria of *H. bacteriophora* coloured the cadaver red, while *S. yirgalemense* coloured the cadaver yellow. No red cadavers were observed in any of the field trials. In Table 3.1, the soil analysis indicates the difference existing in the soil properties between the two trial sites.

Table 3.1: Chemical and physical soil analysis of samples taken from Welgevallen and Nietvoorbij vineyards in Stellenbosch.

| Vineyard | EC (mS/m) | pH (KCl) | C (%) | N (%) | Clay (%) | Silt (%) | Sand (%) | Classification | C:N Ratio |
|---|---|---|---|---|---|---|---|---|---|
| **Nietvoorbij** | 10 | 5.8 | 0.25 | 0.03 | 23 | 18 | 59 | Lm* | 8.33 |
| **Welgevallen** | 22 | 6.0 | 0.80 | 0.09 | 21 | 14 | 65 | SaKlLm** | 8.88 |

*Loam; **Sand/Clay/Loam

*Effects of imidacloprid on survival and virulence*

Data analysed with a three-way ANOVA showed no significant interaction between batches (2 levels: batch 1 and batch 2, used on separate dates), time (4 levels: 0 h, 6 h, 12 h, and 24 h) and treatments (2 levels: Confidor® and water) ($F_{(3, 64)} = 0.0232$; $P = 0.995$). Data from the two batches were pooled, and a two-way ANOVA comparing the treatments and the hours of exposure showed no significant difference in IJ mortalities ($F_{(3, 72)} = 0.048670$; $P = 0.692$) (Fig. 3.3).

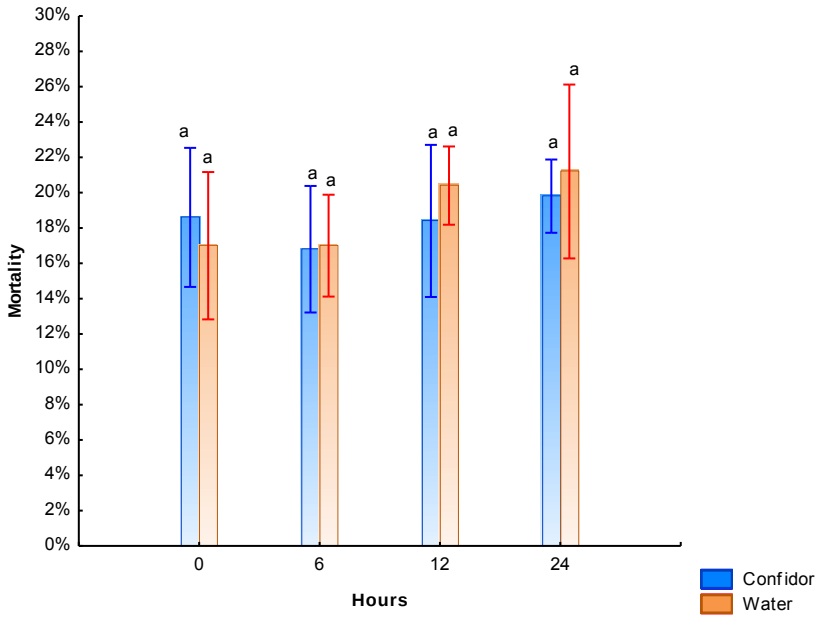


Fig. 3.3: The mean percentage (95% confidence interval) mortality of commercial *Heterorhabditis bacteriophora* IJs after 0, 6, 12, and 24 h exposure to imidacloprid (two-way ANOVA; $F_{(3, 72)} = 0.048$; $P = 0.69$). Bars sharing a common letter are not significantly different.

Steinernema feltiae *survival, over a 24 h exposure period, to imidacloprid*

Data analysed with a three-way ANOVA showed no significant interaction between batches (2 levels: batch 1 and batch 2, used on separate dates), time (4 levels: 0 h, 6 h, 12 h, and 24 h), and treatments (2 levels: imidacloprid and water) ($F_{(3, 64)} = 0.19143$; $P = 0.901$). Data from the two batches were pooled, and a two-way ANOVA comparing the treatments and hours of exposure showed no significant difference in IJ mortalities ($F_{(3, 72)} = 0.6263$; $P = 0.6$) (Fig. 3.4).

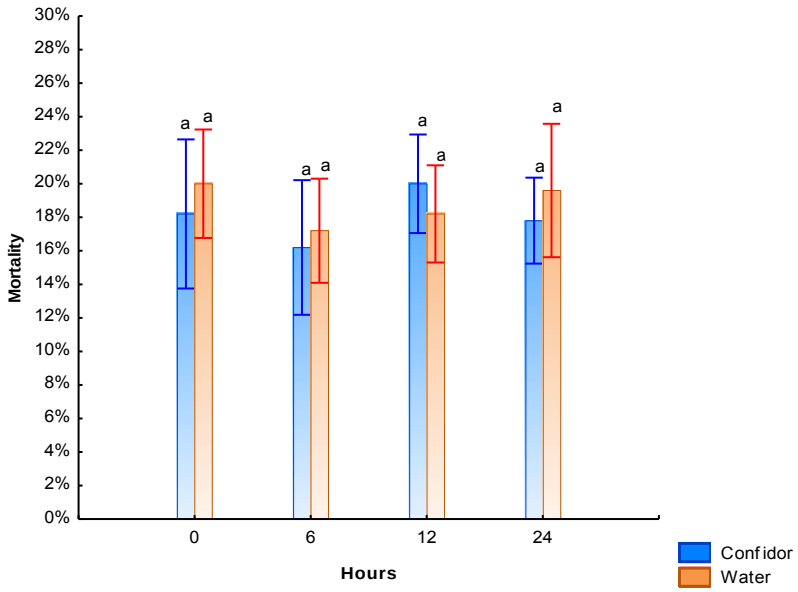


Fig. 3.4: The mean percentage (95% confidence interval) mortality of commercial *Steinernema feltiae* IJs after 0, 6, 12 and 24 h exposure to imidacloprid (two-way ANOVA; $F_{(3, 72)} = 0.626$; $P = 0.6$). Bars sharing a common letter are not significantly different.

Heterorhabditis bacteriophora *virulence after 24 h exposure to imidacloprid*

A two-way ANOVA showed no significant difference between batches ($F_{(2, 24)} = 3.2632$; $P = 0.055$). Data from the two batches were pooled and analysed using a one-way ANOVA, resulting in a significant difference between mortalities of treatments ($F_{(2, 27)} = 154.19$; $P < 0.001$). A significant difference was found between the water (control) treatment, with no mortality, and the other two treatments. No significant difference was found between the percentage mortality of codling moth larvae (virulence of *H. bacteriophora*) inoculated with *H. bacteriophora* exposed to imidacloprid for 24 h (88% ± 3.8% mortality) and with no previous exposure (88% ± 5.9% mortality) ($P = 1$) (Fig. 3.5).

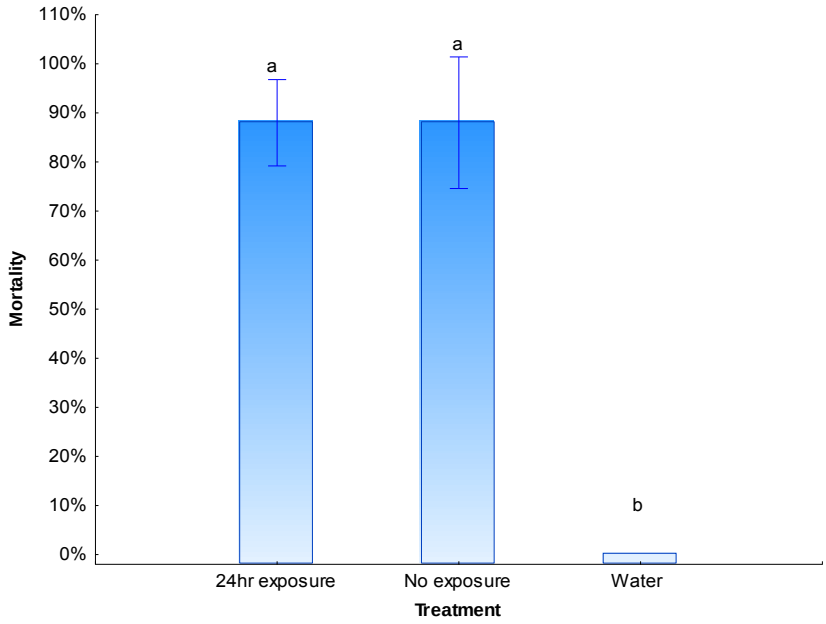


Fig. 3.5: Percentage mortality (95% confidence interval) of *Cydia pomonella* post *Heterorhabditis bacteriophora* 24 h exposure to imidacloprid and no previous exposure (one-way ANOVA; $F_{(2, 27)} = 154.1$; $P < 0.001$). Different letters on bars indicate a significant difference.

Steinernema feltiae *virulence after 24 h exposure to imidacloprid*

A two-way ANOVA showed no significant difference between batches ($F_{(2, 24)} = 2.94$; $P = 0.07$). Data from the two batches were pooled and analysed using a one-way ANOVA, resulting in a significant difference between mortalities in treatments ($F_{(2, 27)} = 196.59$; $P < 0.001$). The significant difference was between the water (control) treatment with zero mortality, and the other two treatments. No significant difference was found between the percentage mortalities of codling moth (virulence of *S. feltiae*) inoculated with *S. feltiae* exposed to imidacloprid for 24 h (82% ± 4.6% mortality) and with no previous exposure (88% ± 3.8% mortality) ($P = 0.1$) (Fig. 3.6).

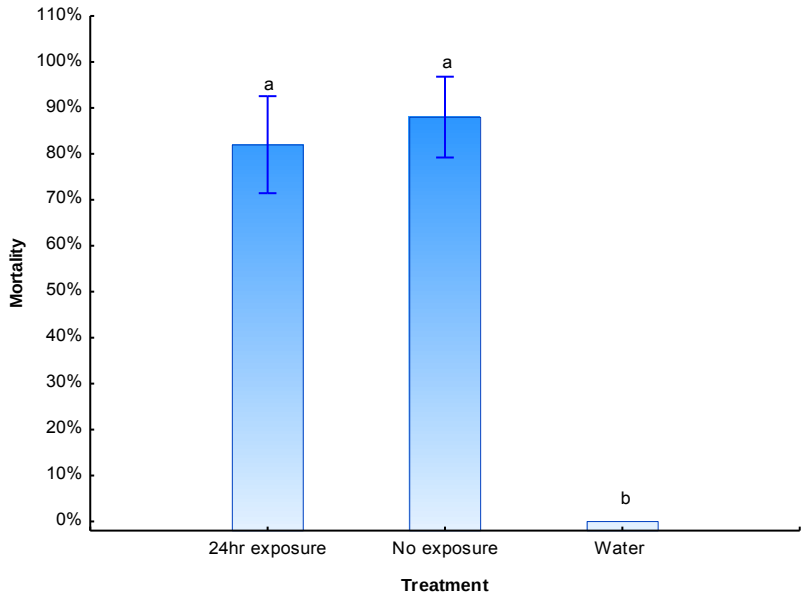


Fig. 3.6: Percentage mortality (95% confidence interval) of *Cydia pomonella* post *Steinernema feltiae* 24 h exposure to imidacloprid and no previous exposure (one-way ANOVA; $F_{(2, 27)} = 196.59$; $P < 0.001$). Different letters on bars indicate a significant difference.

*In-field soil application and infectivity of* Steinernema yirgalemense

*Welgevallen vineyard*

Analysing the data using a one-way ANOVA showed a significant interaction between treatments of *S. yirgalemense* at concentrations of 80, 40, 20, and 0 IJs/cm$^2$ ($F_{(3, 28)} = 7.7252$; $P < 0.001$). *Planococcus ficus* mortalities differed for all treatments compared to the control, with 20 IJs/cm$^2$ obtaining a mean mortality of 23% ± 7% ($P = 0.3$). The concentration of 80 IJs/cm$^2$ was responsible for the greatest *P. ficus* mortality of 50% ± 10% (Fig. 3.7).

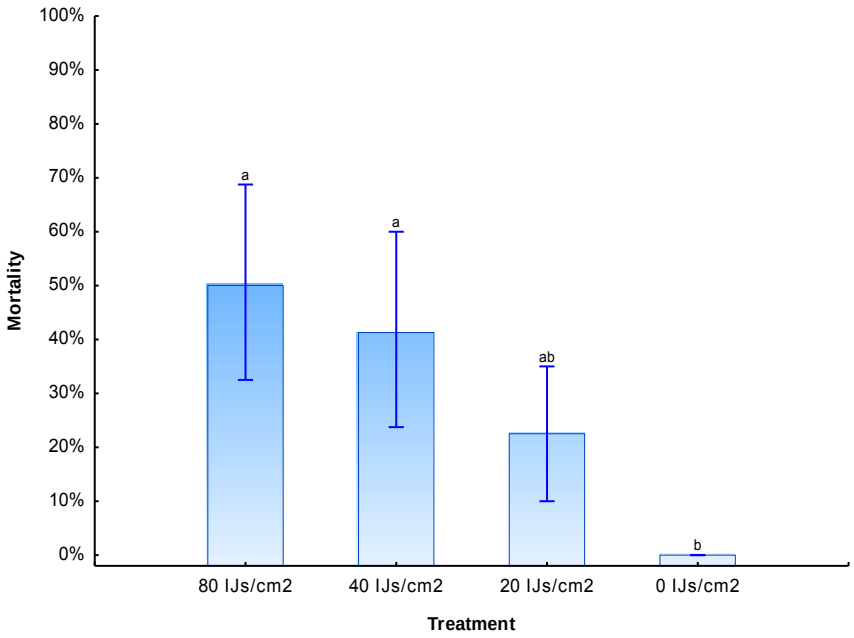


Fig. 3.7: Percentage mortality (95% confidence interval) of *Planococcus ficus* buried 15 cm beneath the soil in the field with a 24 h exposure to *Steinernema yirgalemense* at concentrations of 80, 40, 20, and 0 IJs/cm$^2$ (one-way ANOVA; $F_{(3, 28)} = 7.7252$; $P < 0.001$). Different letters on bars indicate a significant difference.

*Nietvoorbij vineyard*

Data analysed using a one-way ANOVA showed a significant interaction between treatments of *S. yirgalemense* at concentrations of 80, 40, 20, and 0 IJs/cm$^2$ ($F_{(3, 28)} = 5.9448$, $P = 0.0028$). *Planococcus ficus* mortality differed for all treatments compared to the control, while 20 IJs/cm$^2$ obtained a mean mortality of 28% ± 11% ($P = 0.075$).The concentration of 80 IJs/cm$^2$ was responsible for the greatest *P. ficus* mortality of 52% ± 12% (Fig. 3.8).

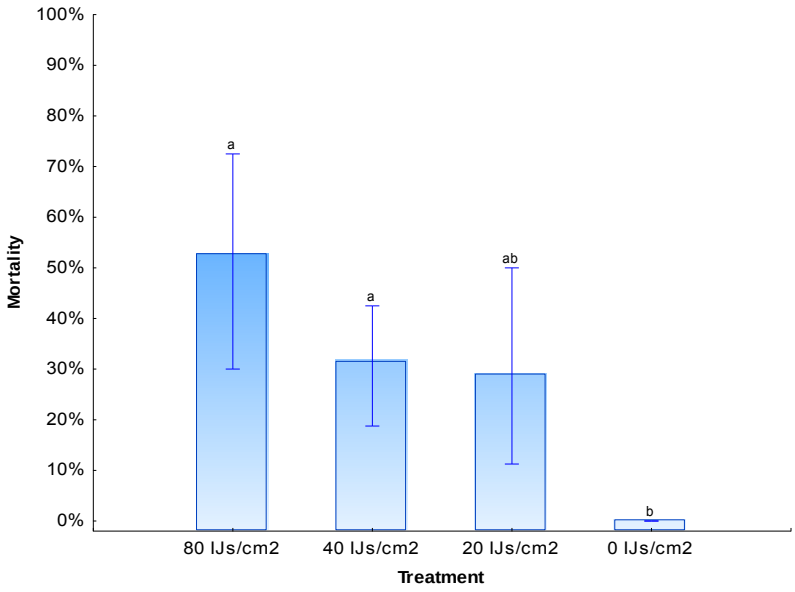


Fig. 3.8: Percentage mortality (95% confidence interval) of *Planococcus ficus* buried 15 cm beneath the soil in the field with a 24 h exposure to *Steinernema yirgalemense* at concentrations of 80, 40, 20, and 0 IJs/cm$^2$ (one-way ANOVA; $F_{(3, 28)} = 5.9448$; $P = 0.0028$). Different letters on bars indicate a significant difference.

*In-field soil persistence of* Steinernema yirgalemense

*Welgevallen vineyard*

A two-way ANOVA comparing IJ concentrations (80, 40, 20 and 0 IJs/cm$^2$) and weeks (1, 2, 4, and 12 weeks) post IJ application, showing no significant interactions when considering codling moth larvae mortality percentages ($F_{(9, 112)} = 0.87904$, $P = 0.546$) (Fig. 3.9).

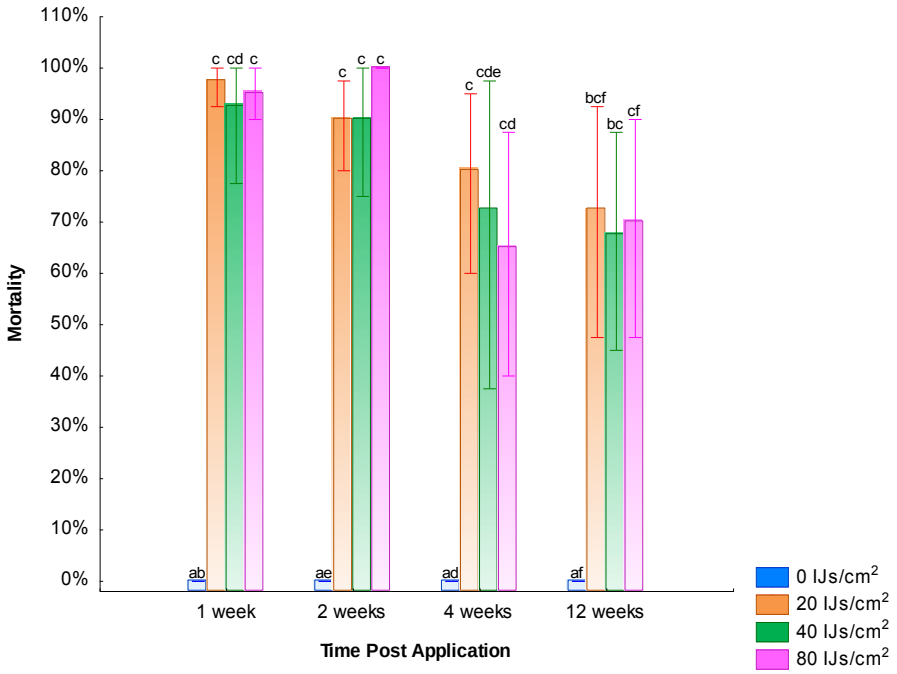


Fig. 3.9: Percentage mortality (95% confidence interval) of *Cydia pomonella*, at Welgevallen, buried 15 cm beneath the soil in the field with a 24 h exposure to *Steinernema yirgalemense* at concentrations of 80, 40, and 0 IJs/cm$^2$ after one, two, four and 12 weeks post EPN application (two-way ANOVA; $F_{(9, 112)} = 0.87904$; $P = 0.546$). Bars sharing a common letter are not significantly different.

*Nietvoorbij vineyard*

Data was analysed using a two-way ANOVA comparing IJ concentrations (80, 40, 20, and 0 IJs/cm$^2$) and weeks (1, 2, 4, and 12 weeks) post IJ application, showing no significant interactions when considering codling moth larvae mortality percentages ($F_{(9, 112)} = 1.5238$; $P = 0.148$) (Fig. 3.10).

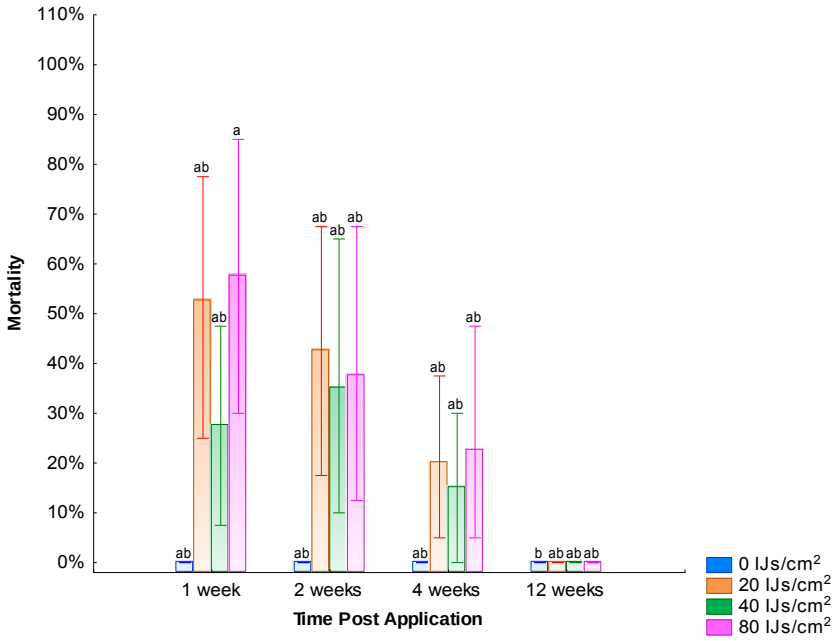


Fig. 3.10: Percentage mortality (95% confidence interval) of *Cydia pomonella*, at Nietvoorbij, buried 15 cm beneath the soil in the field with a 24 h exposure to *Steinernema yirgalemense* at concentrations of 80, 40, 20, and 0 IJs/cm$^2$ after one, two, four, and 12 weeks post EPN application (two-way ANOVA; $F_{(9, 112)} = 1.5238$; $P = 0.148$). Bars sharing a common letter are not significantly different.

*Ant deterrent factors of* Heterorhabditis zealandica *and* Steinernema yirgalemense

A two-way ANOVA comparing EPN species and days post inoculation showed no significant difference in the percentage cadavers remaining intact after 24 h exposure to *L. humile* in the field. Only codling moth larvae cadavers four days post inoculation with *H. zealandica* were significantly different, with 17% ± 1.9% of cadavers still intact, in comparison to the freeze-killed cadavers with zero ($P = 0.03$) (Fig. 3.11).

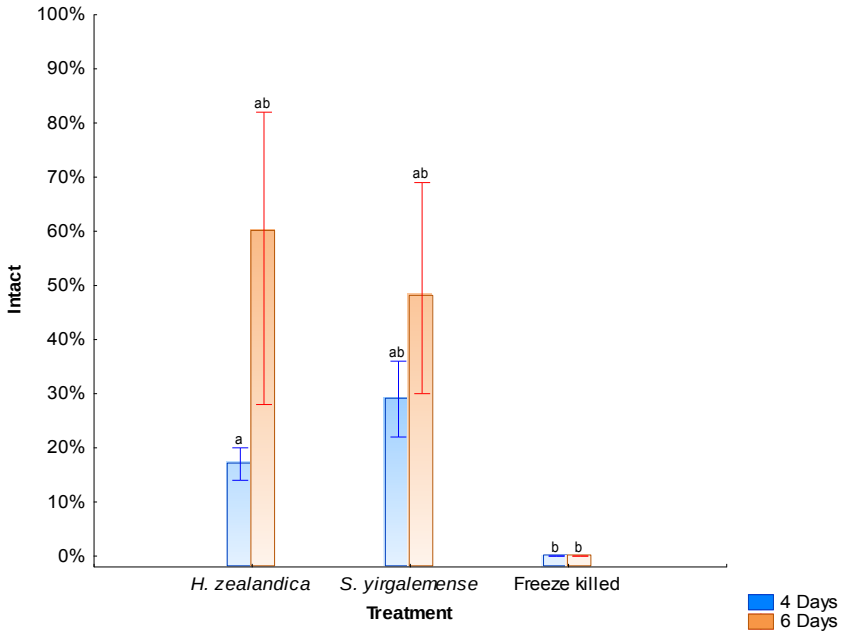


Fig. 3.11: Percentage intact *Cydia pomonella* cadavers (95% confidence interval) of 4 and 6 days post *Heterorhabditis zealandica* and *Steinernema yirgalemense* inoculation after 24 h exposure to *Linepithema humile* (two-way ANOVA; $F_{(2,18)} = 3.1931$; $P = 0.065$). Bars sharing a common letter are not significantly different.

After 24 h exposure to *L. humile*, cadavers infected with *H. zealandica* and *S. yirgalemense* had been completely consumed, bitten, or left intact (Fig. 3.12). For both 4- and 6-day-old cadavers 100% of control cadavers (freeze-killed individuals) were consumed, of the 4-day-old cadavers 15% of *H. zealandica* and 5% of *S. yirgalemense* had been consumed, while 0% of both had been consumed in the 6-day-old *H. zealandica* and *S. yirgalemense* cadavers (Fig. 3.13). Of the 4-day-old cadavers, 17% ± 2% of *H. zealandica* and 29% ± 4.4% of *S. yirgalemense* remained intact, whereas, for the 6-day-old *H. zealandica and S. yirgalemense* cadavers, 60% ± 17% and 49% ± 11% remained intact, respectively (Fig. 3.13).

Fig 3.12:*Cydia pomonella* larvae post 24 h exposure to *Linepithema humile*. The top row is infected with *Steinernema yirgalemense* (with a typical pink/red colour), whereas the bottom row is infected with *Heterorhabditis zealandica* (typically dark brown in colour). The first two cadavers on the left are intact, and the two cadavers on the right have been bitten and left to dry.

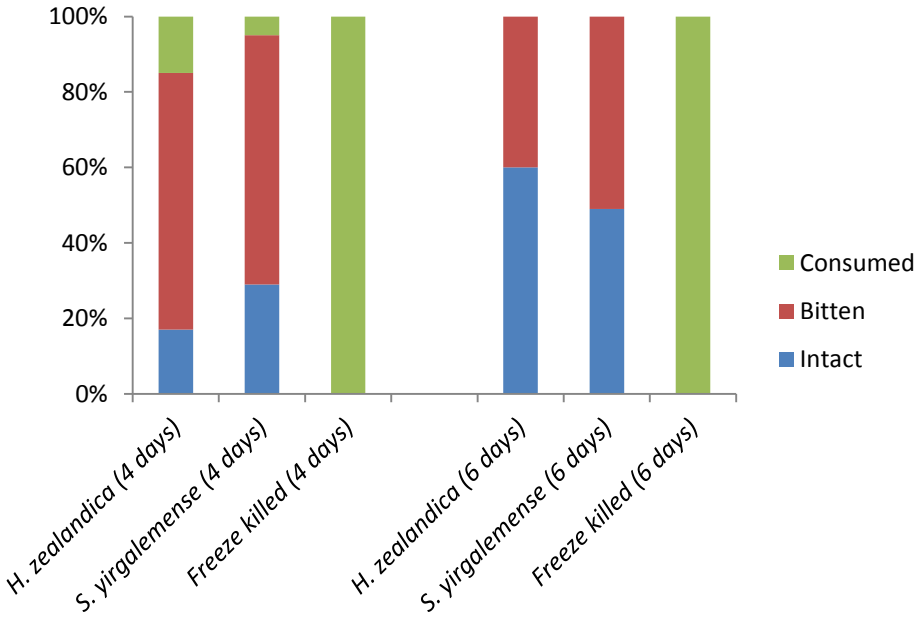


Fig. 3.13: Percentages of 4- and 6-day-old *Cydia pomonella* cadavers infected with *Heterorhabditis zealandica* and *Steinernema yirgalemense* that were consumed, bitten, or left intact after 24 h exposure to *Linepithema humile*.

The control tubes were occupied by *L. humile,* with the cadavers being completely consumed, while tubes of infected, intact cadavers were generally void of ants (Fig. 3.14).

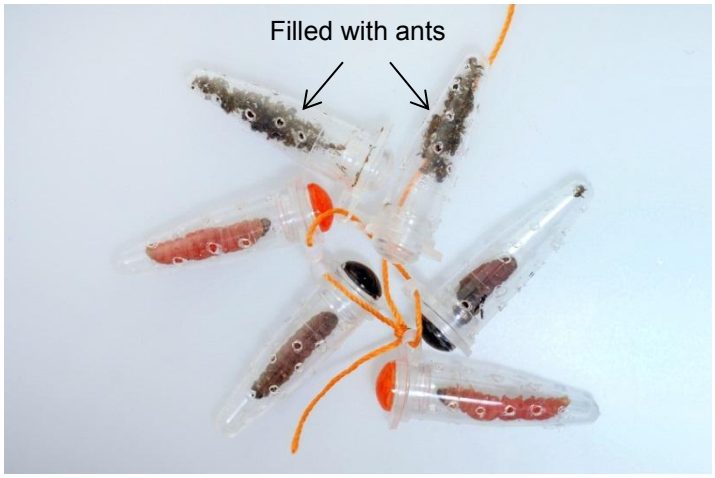


Fig. 3.14: *Cydia pomonella* larvae post 24 h exposure to *Linepithema humile.* Cadavers infected with *Heterorhabditis zealandica* (black caps) and *Steinernema yirgalemense* (orange caps) are intact. Control tubes (clear caps) are filled with ants and cadavers completely consumed.

**Discussion**

The need to test the compatible use of EPNs and agrochemicals is important when considering the use of EPNs in an IPM scheme. Being able to tank-mix the EPNs and the desired agrochemical facilitates the process, and may result in the improved control of a single pest (Koppenhöfer & Grewal, 2005). Many studies have been done to determine the effects of different EPN species in combination with agrochemicals such as insecticides, nematicides and acaricides (Koppenhöfer&Grewal, 2005; Gutiérrez *et al*., 2008; Van Niekerk, 2012).

The tests conducted in this study for commercially produced *H. bacteriophora* and for commercially produced *S. feltiae* concerning both IJ survival and infectivity displayed no negative results. Studies conducted by Alumai and Grewal (2004) and Koppenhöfer *et al*. (2000) found synergistic effects between *H. bacteriophora* and imidacloprid, while Alumai and Grewal (2004) also found that the combination significantly increased the pathogenicity of *H. bacteriophora*. In contrast, a study conducted by Cuthbertson *et al*. (2003) on the combined use of agrochemicals and *S. feltiae* for the control of the sweet

potato whitefly, *Bemisia tabaci* (Gennadius), found that imidacloprid significantly reduced the infectivity of *S. feltiae.* These results are contrasted by those of Koppenhöfer *et al*. (2002), who found the effects to be synergistic when combining *S. feltiae* and imidacloprid when controlling *Exomala (Anomala) orientalis* (Waterhouse), the oriental beetle.

The results obtained in this study could be considered to be additive, instead of synergistic, for both commercially produced *H. bacteriophora* and *S. feltiae* according to Koppenhöfer & Grewal (2005), who describe additive results as being complementary, due to the agents concerned acting independently of each other, as opposed to synergistically, whereby the combination of the agents produce an increased efficacy in control (more so than if the results were simply added together). Based on this data the mixture of the product imidacloprid and of the two EPN species concerned indicates compatibility, enabling tank mixtures to be made for co-application in an IPM programme for grapevines, which should reduce costs and application time.

The two vineyards chosen to test the efficacy of controlling *P. ficus* under the soil in the field using *S. yirgalemense* were analysed separately. In both instances, *S. yirgalemense* performed well, despite the fact that the mealybugs were only left in the soil for 48 h, when, in practice, the nematode will have an indefinite time period for infection. Over and above this, the mealybugs were buried 15 cm beneath the soil, which means that the nematodes had to detect and to infect the insects within a short period of time. These are promising results, when considering that, in South Africa, *P. ficus* spends the winter months in colonies on the lower trunk, under the bark and underground on the roots of the vine, where, subsequently, they can be vulnerable targets to applied EPNs (De Villiers, 2006; Holm, 2008).

To establish the ability of *S. yirgalemense* to persist in the soil post application, codling moth larvae were placed in the soil, due to the difficulty of working with *P. ficus*, due to its small size and sensitivity to handling, which might affect the data, and codling moth larvae known for their susceptibility to *S. yirgalemense* (De Waal *et al*., 2011). The same treatment was applied to both vineyards, and the larvae were left in the soil for 5 days to provide any remaining IJs sufficient time to locate and infect the hosts. Generally speaking, as soon as EPNs are applied to the soil, they are exposed to an array of abiotic and biotic factors that cause nematode mortality (Curren, 1993). Some biotic factors include temperature, soil

texture, and soil moisture, while biotic factors include free-living macro- and micro-fauna and flora, among many others (Curren, 1993; Kaya & Thurston, 1993). Over and above these many factors, some nematode species are known to be characteristically more persistent than are others (Curren, 1993).

The persistence of *S. yirgalemense* on the two vineyards that were used in the current study differed dramatically, with a steady persistence on the Welgevallen vineyard, and a drastic decrease on the Nietvoorbij vineyard over the three months concerned. The difference in the two vineyards could have been due to any of the many abiotic and biotic factors of the two differing vineyards. To hold one factor accountable for the low persistence in Nietvoorbij would be almost impossible, as there might be a multitude of factors at work. To make comparisons, and to draw ideas and conclusions from other persistence studies, is also difficult, as different EPN species are studied under a variety of different conditions. Across studies, many different factors were studied and felt to be responsible for the decline in persistence time, which varied from a few days to a few months (Kung *et al.*, 1990, 1991; Wilson & Gaugler, 2004; Koppenhöfer & Fuzy, 2006, 2007). One possible reason for the sturdy 'persistence' at Welgevallen might be that the IJs could have been recycling, as considered by Curren (1993), thus finding hosts in the soil and continuing with their life cycle, opposed to persisting without available hosts. If this were the case, then the nematodes might establish themselves and, subsequently, improve in controlling the pest insect concerned (Kaya & Stock, 1997). On the other hand the large decline in the percentage mortalities on the Nietvoorbij vineyard between 4 and 12 weeks post application may be due to the fact that some time between those two periods, tillage between the grape vine rows had occurred. Work done by Susurluk and Ehlers (2008) found that ploughing caused a large decline in *H. bacteriophora* persistence. To rule out and to control for certain factors in developing a better understanding of what is affecting the nematodes, persistence tests will need to be done under laboratory conditions.

A factor that had a definite influence on the persistence results, and not necessarily on the actual persistence of the nematodes on the Nietvoorbij vineyard was the presence of ant scavengers. Often the codling moth larvae were found to have been eaten, presumably before the IJs had had the chance to locate the larvae and to infect them. As a result, only the remaining cadavers that had been infected could

contribute to the persistence data. It was found that on control vines (where no IJs had been sprayed) codling moth larvae were missing, while, on the other treatment vines, the larvae remaining were infected. The finding elicited the idea of testing for the possibility of scavenger deterrent factors that might be produced by *S. yirgalemense.* Such studies have been conducted (Baur *et al.,* 1998; Zhou *et al*., 2002; Foltan & Puza, 2009; Gulcu *et al*., 2012), but not on *S. yirgalemense* or *H. zealandica*. Both these nematode species are endemic to South Africa (Malan *et al*., 2006, 2011), and it had been shown that they were both associated with unknown species of *Xenorhabdus* and *Photorhabdus,* respectively (Ferreira, 2013).

Tests concerning the possibility of ant-scavenging deterrent factors would be of great value in this study. Ants involved in symbiotic relationships with mealybugs and other arthropod scavengers pose a threat to EPN population persistence, and might influence the choice of application methods in the field. It is critical that the cadaver infected with EPNs remains intact, to enable nematodes to complete their life cycle (Foltan & Puza, 2009). Invertebrate cadavers are vulnerable to invertebrate scavengers, such as ants, but studies have been conducted to suggest that the nematode/bacteria infestation may possibly deter ants from scavenging (Foltan & Puza, 2009). Zhou *et al*. (2002) used 4-day-old *G. mellonella* larvae killed by different strains of *Photorhabdus luminescens* (Poinar and Thomas) and *Xenorhabdus nematophilus* (Poinar and Thomas), or by freezing, and found that a significantly larger number of non-infected cadavers were removed by the ants concerned. In using 2- and 8-day-old *G. mellonella* larvae infected with different steinernematid and heterorhabditid nematodes, Baur *et al.* (1998) found that different ant species removed more steinernematid-infected cadavers (60-80%) than heterorhabditid-infected larvae (10-20%) within a period of 24 h. Gulcu *et al*. (2012), in furthering the work done by Baur *et al.* (1998) and Zhou *et al*. (2002), extended their tests beyond ants to other arthropod scavengers, such as crickets, wasps and calliphorid flies. They found that the chemical compounds that are produced by the symbiotic bacteria of the EPNs not only deter ants from feeding on the cadavers, but also the insects tested, suggesting that the chemicals be called scavenger deterrent factors, opposed to ant deterrent factors.

Unfortunately, the current study concerning the possible deterrent factors produced by *H. zealandica* and *S. yirgalemense* produced results that displayed no significant difference between the infected cadavers that were bitten and consumed, and those that were left intact. This study should not be neglected, though, as the data suggest, there are factors at work. In every instance, control cadavers that were freeze killed had been entirely consumed, while, in most cases, cadavers that were infected with either *H. zealandica* or *S. yirgalemense* had either been left entirely intact or bitten, but hardly ever entirely consumed. Of the cadavers that were presented 6 days after infection, 60% ± 17% of the cadavers infected by *H. zealandica*, and 49% ± 11% of those infected by *S. yirgalemense* were left intact, which suggests the production of such scavenger deterrent factors. Furthermore, the bitten cadavers may have been 'tested' by the ants and subsequently left alone, due to the deterrent chemicals. However, this still meant that the cadaver dried out, and that the nematodes died. In the soil, where humidity levels are much higher, the cadaver may remain hydrated after a bite, and the nematodes left to complete their cycle.

*Steinernema yirgalemense* produced good results in the current soil field trials, having exposed the sensitivity of soil-dwelling stages of *P. ficus*. Over and above this, the fact that, in the one vineyard, *S. yirgalemense* still persisted, with high mortality of codling moth larvae at three months, was encouraging, when considering the extended period of biopesticidal action that might potentially occur post application. Knowing the compatible use of EPNs with imidacloprid is pertinent in an IPM scheme, as both can be simultaneously applied in the field with confidence, while increasing convenience with co-application and reducing costs for the farmers concerned. These findings, alongside the possible production of scavenger deterrent factors, place *S. yirgalemense* in a promising position as a potential biological control agent for the control of *P. ficus*.

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# CHAPTER 4

## Ecological Factors Affecting Entomopathogenic Nematode Infectivity, Movement and Persistence


### Abstract

Within the environment there are many biotic and abiotic factors that influence the infectivity, movement and persistence of entomopathogenic nematodes (EPNs). Consequently, the efficacy of EPNs, as a biological control agent for the control of the vine mealybug, *Planococcus ficus* (Signoret), is directly affected by such environmental factors. As temperature is one of the most influential factors, laboratory bioassays and vertical soil column tests were conducted, using two South African EPN species, *Heterorhabditis zealandica* and *Steinernema yirgalemense* at 14°C. There was a significant difference in the performance of *H. zealandica* and *S. yirgalemense,* with 2.5% ± 1.2% and 9.1% ± 2.6% mealybug mortality respectively. For the sand column test, at 14°C, *H. zealandica* and *S. yirgalemense* were responsible for insignificantly different mealybug mortalities of 8.5% ± 1.4% and 3.5% ± 2.4%, respectively. These results indicate the low insecticidal activity of the two EPNs species at 14°C. Laboratory persistence trials were conducted, for the two species, to produce data to which field trial data can be compared. Between two and four months the persistence levels of *H. zealandica*, indicated by *Cydia pomonella* larval mortalities, dropped significantly lower than those of *S. yirgalemense* with 44% ± 5.8% and 90% ± 2.9% larval mortalities, respectively, at four months. Results from an olfactometry test indicated a significant difference concerning the number of *S. yirgalemense* infective juveniles (IJs) that were attracted to artificially damaged *Vitis vinifera* roots, (241.8 ± 0.124 IJs) and *P. ficus* (132.5 ± 0.168 IJs), indicating the active movement of the IJs and the attractiveness of organic compounds produced by the roots. This study showed how biotic and abiotic factors can affect EPNs and their abilities as biocontrol agents.


### Introduction

The vine mealybug, *Planococcus ficus* (Signoret), is the dominant mealybug species in South African vineyards (Kriegler, 1954; Walton, 2003; Le Vieux & Malan, 2013a). It is characteristically more

economically damaging than any other mealybug species (Haviland *et al.*, 2005; Daane *et al*., 2008). The vine mealybug displays a clear pattern of vertical seasonal movement on grapevines, where it spends the colder winter months in colonies on the lower regions of the plant, under the bark and underground down to a depth of 30 cm, on vine roots, where it feeds on the plant (De Villiers, 2006; Holm, 2008; Walton & Pringle, 2004).

Entomopathogenic nematodes species of the Heterorhabditidae and Steinernematidae families are lethal insect parasites that have mutualistic associations with the bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Chen *et al*., 2003). When the infective juveniles (IJs) enter into a host, they release their associated bacteria which grow rapidly within the nutrient-rich haemolymph, while producing toxins and other metabolites which kill off the host within 24 to 48 hours after infection (Gaugler *et al*., 1997; Ehlers, 2001; Griffin *et al*., 2005). Consequently, EPNs are used as biological control agents for a range of pests throughout the world (Ehlers, 1996). IJs of both genera use different strategies to find their hosts in the soil and are placed on a spectrum that stretches from 'ambushes', that sit and wait for a passing host to 'cruisers', that actively search for their insect host (Lewis *et al*., 2006; Grewal *et al*., 1994). The capability of IJs to actively disperse through soil and find a host is an important factor which contributes to the success of an EPN species as a biocide (Cutler & Webster, 2003).

Unfortunately, temperature is one of the most important factors restricting the success of EPNs as a biological control agent (Griffin, 1993). It has been found to directly influence pathogenicity (Chen *et al*., 2003; Lacey *et al*., 2006; Molyneux, 1986), survival (Kung *et al*., 1991), movement and host searching (Chen *et al*., 2003; Susurluk, 2008). Two South African studies were conducted, where by De Waal *et al.* (2011) tested the effects of temperature on insecticidal activity of five South African isolates, finding that all the isolates were negatively affected at low temperatures, including *H. zealandica* and *S. yirgalemense*. Van Niekerk and Malan (2012), on the other hand, found the insecticidal capabilities of *H. zealandica* to be unaffected at lower temperatures.

Many studies have been done on nematode persistence in the soil and the influencing factors. Molyneux (1985), Kung *et al*. (1991) and Smits and Wiegers (1991) studied the effects of temperature, Kung *et al*. (1991) investigated the effects of soil humidity levels, while Wilson and Gaugler (2003) found

that populations of mites and Collembola are positively correlated with poor persistence. As a result there are many laboratory and field experiments, all subjected to many different variables, making no situation identical and thus restricting comparability. There are no published studies on South African EPN persistence in the field or under laboratory conditions.

Not much is known about the various searching behaviours of EPNs and the role of chemical communication in the soil, but it is believed that EPNs, of both genera, make use of chemoreception to find their insect hosts (Boff *et al*., 2001; van Tol *et al*., 2001). It has been found that EPNs respond to volatile host associated cues, such as faeces and $CO_2$ (Lewis *et al*., 1993, 2006; Grewal *et al*., 1994). There are also stimuli released by healthy plant roots that may signify a potential habitat for hosts, thus influencing EPN movement and behaviour (Bird & Bird, 1986; Choo *et al*., 1989; Lei *et al*., 1992; van Tol *et al*., 2001). Rasmann *et al*. (2005) and van Tol *et al*. (2001) have identified that plants are not passive victims of herbivorous insects. They have identified a tritrophic level of interaction whereby stimuli emitted from plants damaged by insect herbivory, provide the EPN, *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987, with specific information regarding the presence of potential insect hosts. Rasmann *et al*. (2005) were the first to identify the insect-induced below ground plant signal, (*E*)-β-caryophyllene (released by maize roots), which strongly attracts *H. megidis*. De Waal *et al*. (2011) tested the host seeking ability of six South African isolates, finding that none (including *Steinernema yirgalemense*) displayed a positive attraction to host, besides that of *H. zealandica*.

Le Vieux and Malan (2013b) found *S. yirgalemense* and *H. zealandica* to be the most insecticidal South African EPN isolates for controlling *P. ficus* under optimum laboratory conditions. Both species were also highly effective at infecting and killing *P. ficus* female adults, placed at the bottom of a 15 cm tube filled with sand at 25 ˚C. Extending these studies, the overall objective of the current study was to gain more insight into factors affecting the ecology of *H. zealandica* and *S. yirgalemense.* Specific aims of the study focused on how low temperatures may affect and influence their movement and pathogenicity in the sand, while trying to determine the persistence of the EPNs in sand, under favourable laboratory conditions. Novel work was done to determine the behaviour of *S. yirgalemense* in response to olfactory cues produced by grape vine, *Vitis vinifera,* roots and female adult *P. ficus*.

## Materials and Methods

*Source of nematodes and insects*

IJs were reared at room temperature, using codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae), according to the methods developed by Kaya and Stock (1997). IJs from codling moth cadavers were harvested within the first week of emerging, using White traps (White, 1927). The IJs were stored horizontally in 500 ml vented culture flasks, containing approximately 150 ml of distilled water in a fridge at 14°C. To prevent the use of potentially weak and unhealthy nematodes, the EPNs were used within a month after harvesting. To aid in nematode survival during storage, culture flasks were shaken weekly to increase aeration. The two local EPN species used, namely *H. zealandica* (SF 41) and *S. yirgalemense* (157-C), were originally obtained from previous local surveys and are maintained and stored in the Stellenbosch University nematode collection (Malan *et al.*, 2006, 2011).

A laboratory colony of *P. ficus* was established and reared on butternuts at 25°C, while *C. pomonella* eggs and diet were obtained from Entomon Technologies (Pty) (Ltd), Stellenbosch. These were reared to last – instar larvae under diapausing conditions [photoperiod 10:14 (L: D)], 25˚C, and 60% humidity and were stored in the diet in a closed container in a cold room at 5˚C until needed.

*Effect of low temperature on the mortality of adult females of* P. ficus

For this bioassay 24-well bioassay trays (Flat bottom, Nunc™, Cat. No. 144530, Thermo Fisher Scientific (Pty) Ltd., Gauteng, Johannesburg, South Africa) were used to test the ability of *S. yirgalemense* and *H. zealandica* to infect adult female *P. ficus* at 14°C. To reduce possible edge effects and to ensure an even distribution in the plate, the bottom of each alternate well was lined with a circular (13-mm-diameter) piece of filter paper. Thus only 12 wells were occupied per tray. Five trays were used for each treatment, as well as five trays for the control. A single adult female *P. ficus* was placed in each well and inoculated with 100 IJ/ 50 µl within the 14°C chamber. The *P. ficus* individuals, in the controls, received 50 µl of 14°C water only. To prevent the insects from escaping, each plate was covered with a glass pane inside the lid and held closed with a rubber band. The trays were placed in plastic containers, the lids closed and left in a dark chamber at 14°C for 48 hours. After the two days the number of dead

individuals from the treatment and controls were recorded and infection confirmed by dissection. The procedure was repeated on a separate date with a fresh batch of nematodes.

*Effect of low temperature on vertical movement in sand*

The ability of *S. yirgalemense* and *H. zealandica* to detect and infect adult female *P. ficus* in vertically placed sand columns at 14°C was tested. Five mealybugs were placed in a single perforated PCR 0.2 ml tube. The tube was then placed at the bottom of a 15 × 1.5 cm centrifuge tube. For each nematode species, 20 tubes (therefor in total 100 mealybugs) were used. To sterilise the river sand, it was frozen at -40°C, and dried in an oven overnight at 50°C. To ensure the correct moisture level and temperature, a ratio of 1:10 v/v of water and sand was mixed and left to cool for 12 h at 14°C in the fridge. Each centrifuge tube was filled with the sand to the top. A 13-mm-diameter piece of filter paper was inoculated with 500 IJs/ 50 µl. The inoculated side was placed downward and left on top of the sand. The lid of each centrifuge tube was screwed on and they were placed vertically in a dark chamber at 14°C for 48 h. After the two days dead individuals from the treatment and controls were recorded. The experiment was repeated on a separate date with a fresh batch of nematodes.

*Laboratory persistence of* S. yirgalemense *and* H. zealandica

Sterilised river sand, prepared at a ratio of 1:10 v/v of water and sand, was added to 250 ml plastic tubs and each tub was then inoculated with 10 IJ/cm$^2$, which equated to 1040 IJs a tub that were placed at the centre of each tub. This was done for both *S. yirgalemense* and *H. zealandica.* Once the tubs were inoculated they were then placed in a large plastic container, lined at the bottom with moist paper, closed and left in a room at 25°C for the duration of the experiment. Baiting was conducted by using ten codling moths per tub at 0, 1, 2, 3 and 4 months after initial IJ inoculation. Each bating session comprised of two batches with five tubs for each batch, for both species and the control tubs (which received no IJs), thus a total of 30 tubs. The codling moth larvae in the tubs were left in a dark growth chamber at 25 ± 2°C for 48 h. Dead codling moth larvae were immediately dissected and checked for infection, while living individuals were rinsed and left in a dark growth chamber at 25 ± 2°C for another 48 h and then checked for

infection, if they were dead. The experiment was repeated on a separate date with a fresh batch of nematodes. Data from the two batches were then pooled and analysed.

*Cue attraction response for* S yirgalemense

A three armed olfactometer was assembled. Three small holes were made in the sides of a 7-cm-diameter petri dish and one small hole, to fit the tip of an Eppendorf pipette tip, in the centre of the lid. Three disposable plastic pipettes were cut to remove the centre column; the front 4 cm and the bulb were then re-connected. The petri dish and the three modified pipettes were filled with sterilised river sand, prepared at a ratio of 1:10 v/v of water and sand. The lid was placed on the petri dish and sealed with PARAFILM®. The tips of the three pipettes were inserted into the holes of the petri dish. Of the three bulbs one contained nothing (control), the other 15 *P. ficus* adults and the third 0.5 g of *V. vinifera* roots, collected from a grapevine and cut into small pieces (Fig. 4.1). The experimental setup, containing the study subjects, was left for 6 h to allow a chemical gradient to develop in the sand. Approximately 2000 IJs of *S. yirgalemense,* concentrated in 100 µl water, were pipetted through a small hole made in the centre of the lid of the petri dish after which the hole was sealed using BostikPrestik®. The apparatus was left in a dark growth chamber at 25 ± 2°C for 24 h, after which the contents of each pipette arm were emptied separately into a petri dish, the mealybugs was removed, followed by rinsing the inside of the arm and bulb. Water was added and the IJs were counted with the aid of a Leica stereomicroscope. The mealybugs were then placed on moistened filter paper and left for a further 48 h in the incubator, after which each was dissected for developed nematodes, which were counted and added to the total, found in the olfactometer arm.

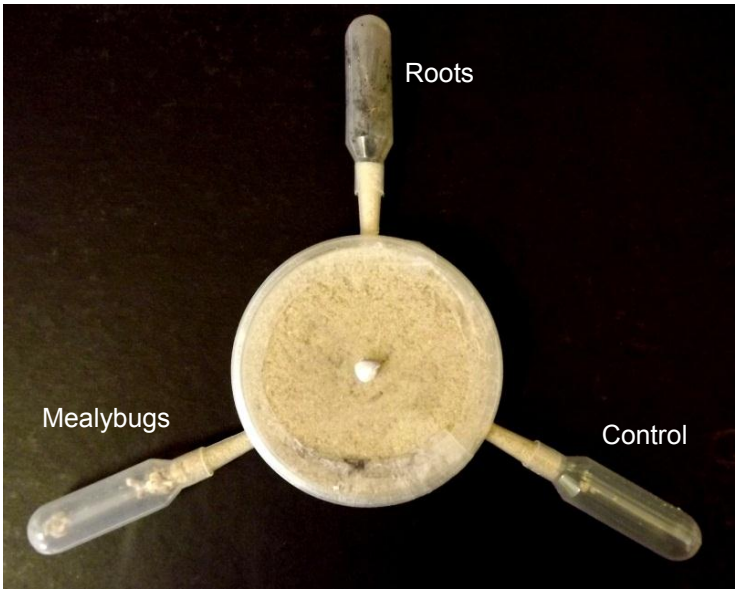


Fig 4.1: Assembled three armed olfactometer with arm bulbs containing mealybugs, vine roots and an empty arm bulb that served as control. A total of 2000 IJ/100 µl water of *Steinernema yirgalemense* were pipetted into the centre hole of the petri dish and resealed.

*Data analysis*

All statistical analyses were done using STATISTICA version 11 (StatSoft Inc. 2012). An analysis of variance (ANOVA) was used to analyse the data. A post-hoc comparison of means was done using Bonferroni's method with 95% confidence intervals (Efron & Tibshirani, 1993). Data from different test dates were pooled and then analysed. For the olfactometer test an ANOVA was done using a generalised non-linear model (GLZ), assuming a Poisson distribution of the variables (counting variables) with a log link function. The Wald test was used as the test statistic.

**Results**

*Effect of low temperature on the mortality of adult females of* P. ficus

Results obtained for mortality of *P. ficus* at 14 °C were pooled and analysed using a one-way ANOVA. A significant difference was obtained in the overall interaction between treatments ($F_{(2, 27)} = 7.93$; $P = 0.001$). There was a significant difference in mortality of *P. ficus* between *S. yirgalemense* (9.1% ± 2.6%) and the control ($P = 0.001$) and between *S. yirgalemense* and *H. zealandica* ($P< 0.05$). However, no

significant difference was found between the percentage mortalities of *P. ficus* in the control and those inoculated with *H. zealandica* (2.5% ± 1.2%) (*P* = 0.9) (Fig. 4.2).

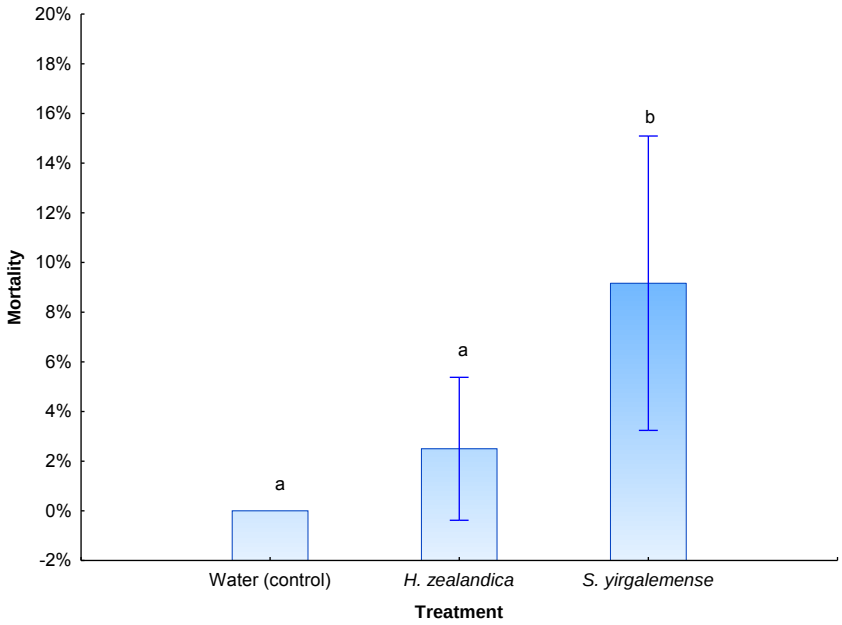


Fig. 4.2: The mean percentage mortality (95% confidence interval) of adult female *Planococcus ficus* inoculated with *Steinernema yirgalemense* and *Heterorhabditis zealandica* at a concentration of 100 IJs/50 µl/insect at 14˚C after 48 h (one-way ANOVA; $F_{(2, 27)} = 7.93$; *P* = 0.001). Bars sharing a common letter are not significantly different.

*Effect of low temperature on vertical movement in sand*

Pooled data obtained from the bioassay determining the vertical movement at low temperatures, were analysed with a one-way ANOVA. Significant differences were obtained over all interactions between the treatments ($F_{(2,117)} = 6.767$; *P* = 0.001). There was a significant difference between mortality percentages of the control and mealybugs inoculate with *H. zealandica* (*P* = 0.001). There was no difference between mealybug mortalities between by *H. zealandica* (8.5% ± 1.4% mortality) and *S. yirgalemense* (3.5% ± 2.4% mortality) (*P* = 0.1) and *S. yirgalemense* and the control (Fig. 4.3)

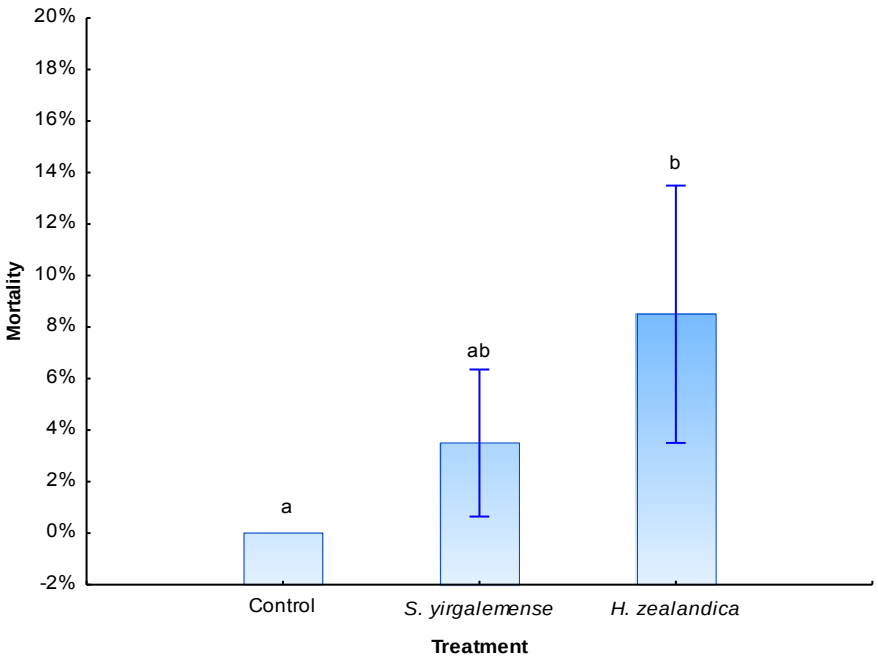


Fig. 4.3: The mean mortality (95% confidence interval) of female adult *Planococcus ficus* buried under 15 cm of sand after inoculation with *Heterorhabditis zealandica* and *Steinernema yirgalemense* at a concentration of 100 IJs /mealybug at 14˚C after 48 h (one-way ANOVA; $F_{(2, 117)} = 6.767$; $P = 0.001$). Different lettering indicates significant differences.

*Laboratory persistence of* S. yirgalemense *and* H. zealandica

Results obtained from testing the laboratory persistence of the two species were pooled and analysed with a two-way ANOVA. Significant overall interactions between the species (2 levels: *H. zealandica* and *S. yirgalemense*) and months (5 levels: 0, 1, 2, 3, 4 months) ($F_{(4, 90)} = 18.342$; $P = < 0.001$) were obtained. There was no significant difference in mortalities over the five dates for *S. yirgalemense.* By the fourth month the percentage mortality caused by *H. zealandica,* differed significantly from those of *H. zealandica* in proceeding months and the mortalities caused by *S. yirgalemense* at the fourth month ($P< 0.001$). From 0 months to 4 months, *C. pomonella* mortalities due to *H. zealandica* were reduced from 100% ± 0% to 44% ± 5.8% (Fig. 4.4).

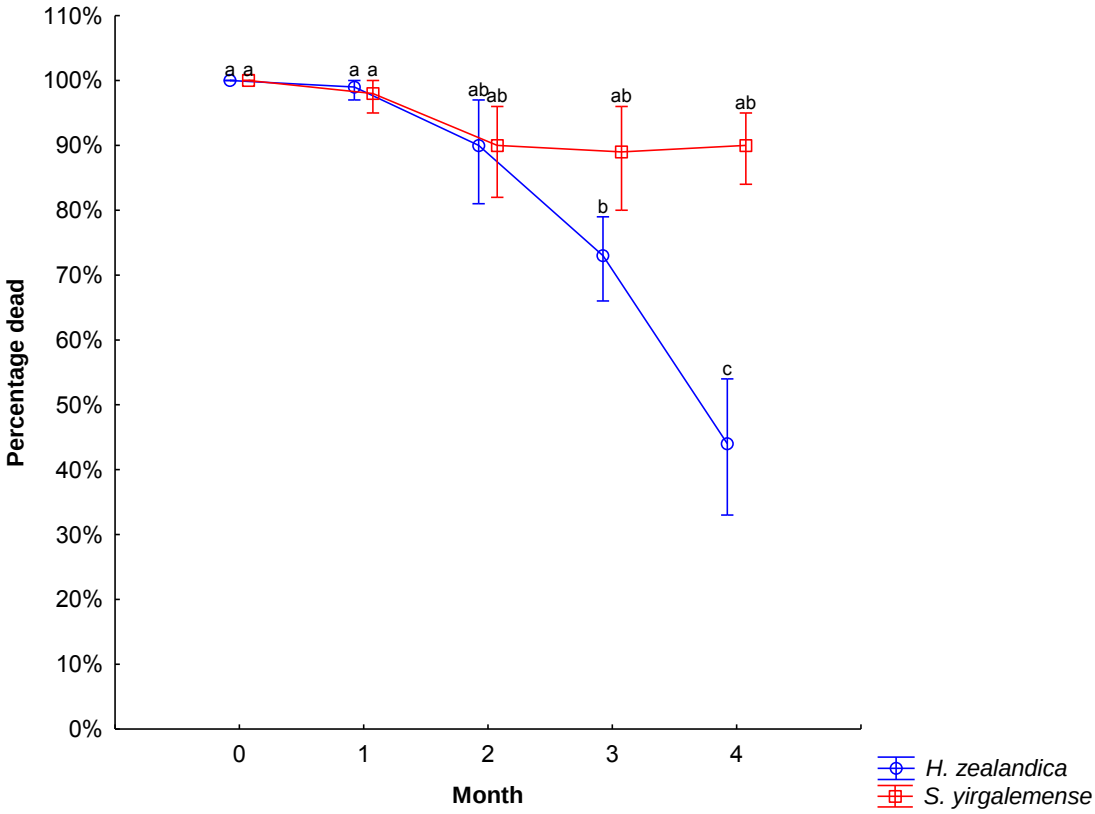


Fig. 4.4: Percentage mortality (95% confidence interval) of *Cydia pomonella* exposed to 10 IJ/cm$^2$ *Heterorhabditis zealandica* and *Steinernema yirgalemense* IJs after 0, 1, 2, 3, and 4 months persistence in sand (one-way ANOVA; $F_{(4, 90)} = 18.342$; $P < 0.001$). Different letters on bars indicate a significant difference.

*Cue attraction response for* S. yirgalemense

From the results obtained on the movement of the IJs towards the three different arms of the olfactometer, no significant difference was found between the three batches ($P = 0.49$). Data from batches were pooled and an analyses was done using a one-way ANOVA, which showed overall significant interactions between treatments (Wald $X^2$ (2) = 23.363; P < 0.001). Significant differences were evident between the average numbers of IJs found having moved towards the control 3.5 ± 1.02 IJs, the grape vine roots 241.8 ± 0.124 IJs and the mealybugs 132.5 ± 0.168 IJs (P < 0.05) (Fig.4.5).

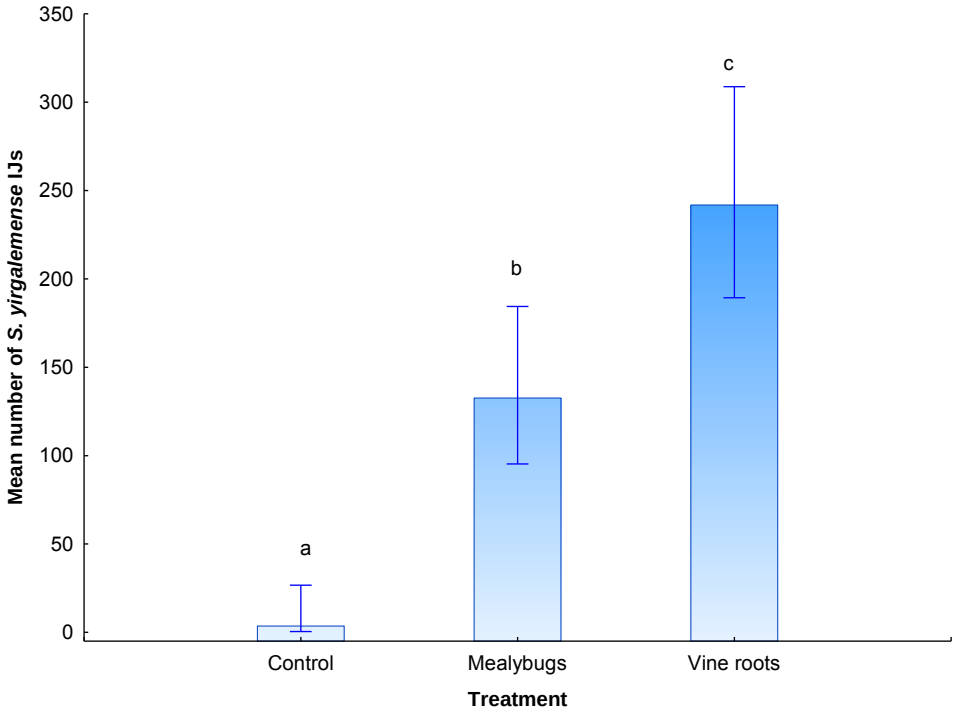


Fig. 4.5: The mean number of *Steinernema yirgalemense* infective juveniles recovered from olfactometer arms connected to bulbs containing either adult *Planococcus ficus* females, grapevine roots or nothing, 24 h after innoculation (one-way ANOVA; Wald $X^2$ (2) = 23.363; P < 0.001) (95% confidence interval). Different lettering on bars indicates a significant difference.

## Discussion

When testing the viability of an EPN species as a potential biocontrol agent, its performance under various conditions need to be investigated. One factor in particular that is known to affect EPNs in various ways is temperature. In northern Europe and other temperate regions, cooler temperatures (< 15°C) are a major limiting factor preventing the use of EPNs as a biocontrol agent (Long, *et al*., 2000). Many authors have investigated the effects of a range of temperatures on various EPN species (Molyneux, 1986; Long, *et al*., 2000; Lacey *et al*., 2006; De Waal *et al.,* 2011; Van Niekerk & Malan, 2012). Different species tend to perform better at different temperatures, with the general consensus that *Steinernema feltiae* can still perform well at temperatures below 15˚C (Molyneux, 1986; Lacey *et al*., 2006; Susurluk, 2008).

Taking this into consideration, both the abilities of *S. yirgalemense* and *H. zealandica* to move and infect hosts at 14˚C were tested. At 25˚C *S. yirgalemense* and *H. zealandica* caused mealybug mortalities of 65% ± 10% and 96% ± 2% in the bioassay, respectively, and 95% ± 1.4% and 82% ± 4.1% mealybug mortality in the sand column test, respectively (Le Vieux & Malan, 2013b). The mortalities at 14˚C for *S. yirgalemense* and *H. zealandica* were found to be 9% ± 2.6% and 2.5% ± 1.2%, respectively and the vertical sand column test for *S. yirgalemense* and *H. zealandica* produced low mealybug mortalities of 3.5% ± 2.4% and 8.5% ± 1.4%, respectively. This drastic decrease illustrates the low infectivity of the two local species at low temperatures.

Authors such as Molyneux (1986), Lacey *et al*. (2006) and Susurluk (2008) all support such findings, having found a decrease in movement and pathogenicity of different EPN species at lower temperatures. De Waal *et al*. (2011) tested five South African EPN species (including *H. zealandica* and *S. yirgalemense*), of which all showed decreased larvicidal activity towards *Cydia pomonella* larvae at 17 ˚C and 12 ˚C. On the contrary Van Niekerk & Malan (2012) found the insecticidal activity of *H. zealandica* to be unaffected by lower temperatures, such as 15˚C. The results of the current study could indicate potential difficulties in the efficacy of *H. zealandica* or *S. yirgalemense* in the field, when targeting overwintering mealybugs. This does not go to say that once temperatures rise to a favourable level, the EPNs will not become active and effective again, as the fore mentioned and tested temperature is not lethal to the two EPNs species concerned. One other factor that may improve the results of the current tests would have been to increase the time given to the EPNs to move, infect and kill the mealybug hosts.

There is numerous amounts of data on the persistence of EPNs, but the parameters for each differs, concerning the strains and environmental conditions tested (Smits, 1996). As a result a laboratory persistence trial for both *H. zealandica* and *S. yirgalemense* was conducted in the hope that comparable results could be produced for current and future studies. When investigating the persistence of nematodes in the field (Chapter 3), results could possibly be influenced by the availability of insect hosts (Susurluk & Ehlers, 2008). Considering this for example, in-field persistence studies will not be able to distinguish between the persistence of the release population of EPNs and the continuation of offspring from the released population, otherwise known as recycling (Curren, 1993; Susurluk & Ehlers, 2008).

Favourable laboratory conditions were set up to establish the persistence in such conditions, providing a 'model' for the comparison of other results. Although the current laboratory persistence trials only extend to 4 months, it is long enough to draw some conclusions. The use of codling moth mortality is an indirect measurement of persistence. To gain precise data on persistence, the original numbers of living IJs that were used to inoculate the containers would need to be known and at a given time the number of live individuals would need to washed from the medium and be counted. Such a process would be laborious and impractical to conduct. The decrease in the percentage codling moth larvae mortality between 2 and 4 months as a result of *H. zealandica* infection, indicates a reduction in the number of IJs persisting in the container. On the other hand there was still a steady persistence for *S. yirgalemense* at 12 weeks, inferring its superiority to *H. zealandica* in its ability to persist. When comparing these results to those found in the field trials (Chapter 3), it is evident that environmental conditions (be they abiotic, biotic and/or anthropogenic) at Nietvoorbij were less conducive to *S. yirgalemense* IJ persistence than at Wellgevallen. Not having counted the number of IJs present in the soil, means that the longevity of the different EPN species over time could not be determined, but nevertheless indications of persistence and insecticidal activity were demonstrated.

Despite the current variations in-field persistence studies and the differing techniques in laboratory persistence studies, contemporary work has been done that allows for reliable identification and quantification of microorganisms in the soil. The use of quantitative real-time PCR (qPCR) can be used in the field and in the laboratory for identifying and quantifying EPN species (Campos-Herrera *et al*., 2011; Campos-Herrera *et al*., 2011). Conventional insect baiting techniques can now be replaced by the qPCR method, which is faster, more accurate and affordable (Campos-Herrera *et al*., 2011). Such a technique should subsequently be used for persistence trials.

According to Hunter (2001) there is a large gap in the understanding of below-ground herbivory and how roots are able to gain protection from herbivory through chemical defence mechanisms. As of such, studies suggest that the defence mechanisms of roots may be the same as in above-ground tissues (Hunter, 2001). There is increasing information that roots secrete chemicals that initiate dialogue between the plant roots and soil microbes such that infested plants have the ability to attract predators of the pest,

in a 'cry for help' (Hampel *et al*., 2005; Badri & Vivanco, 2009). Communication with the surrounding environment is done by utilising the release of volatile organic compounds. The volatile compounds mostly responsible for defence and resistance activities are known as terpenes, of which its biosynthesis by roots and function is an area that has been relatively unexplored (Hampel *et al*., 2005; Tholl, 2006).

*Vitis vinifera* is susceptible to many pathogens and is known to show few induced responses to their attack (Kellow *et al*., 2004). Recently, Lawo *et al*. (2011) conducted the first comparative study on the production of volatile metabolites by uninfested and grape phylloxera, *Daktulosphaira vitifoliae* (Fitch), infested root tips of the grapevine. Nine terpenes were identified to be metabolites of the vine root, of which beta-caryophyllene was produced due to herbivore attack (Lawo *et al*., 2011). Considering that phylloxera is part of the Hemiptera, such as *P. ficus*, it is likely that the feeding of *P. ficus* on the vine roots will also initiate the production of beta-caryophyllene. It is known that beta-caryophyllene produced by insect herbivory on maize roots, strongly attracts the IJ of *H. megidis* (Rasmann *et al*., 2005).

The three armed olfactometer designed in this study, showed that artificially damaged *V. vinifera* roots attracted a significantly larger number of *S. yirgalemense* IJs than that of *P. ficus* adult females. This test suggests two important ecological factors concerning *S. yirgalemense.* One is that it actively responds to host cues and moves towards the source of the organic compounds, which opposes the findings of De Waal *et al*. (2011) and secondly that it has a greater attraction to volatiles produced by roots than that of the herbivore, *P. ficus*. In this instance it is fair to deduce that *V. vinifera* could be involved (under natural conditions) in tritrophic interactions involving EPNs and insect herbivores such as *P. ficus.*

In this study the ability of *H. zealandica* and *S. yirgalemense* to perform as biological control agents at low temperatures was shown to be clearly compromised. Fortunately, at temperatures <14 °C the survival of the EPNs is not hampered, but their activity is only delayed until temperatures return to favourable levels. The persistence of *S. yirgalemense* in the laboratory indicates how in the field the various environmental factors can influence its persistence. For each farmer who considers the use of EPNs, they would need to follow up after their soil application and test for the persistence of the EPNs to gain insight into the frequency of EPN application needed to sustain the required levels of control on their specific farm. The great importance of the olfactometry test is that it is now clear that *S. yirgalemense* can detect

host and plant cues and will seek out both in the search for a new host. It is also encouraging to know that *V. vinifera* roots produce compounds under stress to which *S. yirgalemense* responds actively.

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# CHAPTER 5

## Conclusion

The overall aim of this thesis was to generate information to assist in the control of vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) in South African wine and table grape vineyards by using entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae and Steinernematidae). The main objectives of the study were to initially establish which species and isolates of EPNs would be the most effective in controlling adult female *P. ficus* in the laboratory. This was established by using six local EPN species and two commercially available species. Secondly the compatibility of the two selected EPNs with the agrochemical, imidacloprid (Confidor®), to which the EPNs would very likely be exposed to in an IPM programme, was evaluated. The third objective was to establish the efficacy of the selected EPN to control subterranean *P. ficus* on the roots of grapevines in the field. Lastly various ecological factors were tested to establish their different effects on the efficacy of EPNs as a biological control agent.

The first section of the study focussed entirely on laboratory work, whereby six South African nematode species and two commercially available species were screened to identify the two candidates, which show the greatest potential for controlling *P. ficus. Heterorhabditis zealandica* (SF 41), Poinar, 1990 and *Steinernema yirgalemense* (157-C) Tesfamariam, Gozel, Gaugler & Adams, 2004 produced the greatest mealybug mortalities, out competing the two commercially produced species. This reason alone should discourage the use of exotic nematode species and encourage research and funding directed towards commercial production of local, South African EPN species. From these results, *H. zealandica* and *S. yirgalemense* were then selected for further studies in the laboratory. Both were able to complete their life cycles inside adult female *P. ficus* cadavers and both were able to move through sand columns, infect and kill *P. ficus* adults at 25°C. An EPN concentration trial showed a definite positive relationship that exists between the concentration of the nematode species used for inoculation and the percentage mortality of *P. ficus*. From this study the $LC_{50}$ and $LC_{90}$ values were established, where *H. zealandica* and *S. yirgalemense* produced similar, competitive results. The results obtained from the laboratory bioassays

done in Chapter 2, illustrated the great potential of both the selected EPN species for controlling *P. ficus* in the field.

In Chapter 3, objectives concerning the compatible use of EPNs and agrochemicals as well as the field performance of *S. yirgalemense,* for controlling *P. ficus* adult females underground were approached. Both the commercially produced EPNs *Steinernema feltiae* and *Heterorhabditis bacteriophora* indicated no negative side effects related to the pesticide imidacloprid. Neither the nematode's survival nor virulence was compromised. This is encouraging as tank mixing EPNs and agrochemicals is convenient and helps to save farmers time and money when applying the two products together in an IPM scheme. The two vineyards chosen, Welgevallen and Nietvoorbij, to conduct the field trials were characteristically different. The Welgevallen vineyard is found at the foothills of the Stellenbosch mountains, protected from the wind and comprised of dark moist soil, while Nietvoorbij, on the other hand, is situated at the top of a hill, exposed to the characteristic strong South easterly and North westerly winds and has light coloured, rocky dry soil. The differences of the two vineyards did not affect the mean mortality results of 50% ± 10% at Welgevallen and 52% ± 12% at Nietvoorbij of adult female *P. ficus* mortalities at a concentration of 80 IJs/cm$^2$. Such results are promising when considering that the mealybugs were buried 15 cm below the soil surface and were only given 48 h to detect and infect them. Differences for the two vineyards came about when testing the persistence of *S. yirgalemense*. At Nietvoorbij there was no indication of the nematode still persisting after 12 weeks, while at Welgevallen the codling moth larval mortalities (indicative of *S. yirgalemense* persistence) were still above 70% at 12 weeks post application, indicating good persistence and insecticidal activity. Such a test illustrates the uniqueness of each farm and how different conditions will affect EPNs in either positive or negative ways.

Inadvertently an experiment developed through the findings of the field persistence trials, which was to determine whether or not *H. zealandica* and *S. yirgalemense* were able to produce scavenger deterrent factors that would protect the infected cadavers in the field. The argentine ant, being a very predominant species in the Western Cape vineyards, was selected at the scavenger arthropod. Unfortunately no significant results in the experimental setup were produced, but a clear trend was visible illustrating that

these two EPN species may produce deterrent factors. However, control cadavers (freeze killed) were all entirely consumed while 60% ± 17% of *H. zealandica* and 49% ± 11% of *S. yirgalemense* infected cadavers (6 day old) were left intact. The value in such a study lies with the fact that if *H. zealandica* and *S. yirgalemense* produce such insect deterrent factors, then their ability to recycle and persist in the applied vineyard will increase, thus increasing their efficacy as a biological control agent.

The last objective, of establishing the effects of environmental factors on the efficacy of EPNs as a biological control agent for *P. ficus*, involved repeating the bioassay and sand column tests in the laboratory, but at 14°C instead of at 25°C. The results for both species for both tests indicated the low efficacy of both species to perform at this temperature. The results of the study could indicate potential difficulties of controlling overwintering mealybugs in the field. This does not, however, mean that the EPNs are killed at this temperature, but that once temperatures rise to favourable levels the EPNs will become active once again.

The extensive data available on the persistence of applied EPNs involves many different parameters for each species, when concerning the strains and environmental conditions tested, making the comparison of results difficult. The laboratory persistence trial for both *H. zealandica* and *S. yirgalemense* was conducted in the hope that comparable results would be produced for current and future South African studies and to help draw conclusions from the infield persistence trials conducted in Chapter 3. For example, field persistence studies were not able to distinguish between the persistence of the release population of EPNs or the recycling and continuation of their offspring through available hosts. The laboratory persistence trials indicated a drop in the levels of *H. zealandica* after four months, while *S. yirgalemense* still continued steadily, which was a similar finding at the Welgevallen vineyard, but not at Nietvoorbij. Reasons could range from the Nietvoorbij vineyard being so exposed to the winds and sun, the presence of many arthropod scavengers, the soil type and possibly the management practices applied by the farmer.

Other environmental factors that affect nematodes are those of a biotic nature. In this instance plants are able to produce volatile organic compounds which serve to communicate with other surrounding plants and microorganisms. The three-armed olfactometry test established that both *P. ficus* and

damaged grape vine roots produce chemicals that are attractive to *S. yirgalemense*. The test also confirmed that *S. yirgalemense* actively moves towards the source of these chemicals. Over and above these two findings, damaged vine root attracted a significantly higher number of IJs than that of the mealybugs. This indicates that there may be tritrophic levels of interaction between the plant, insect pests and EPNs in the field. Thus the grapevine roots are not a passive victim to insect herbivores, but instead utilises EPNs by attracting them to the insect pest.

Unfortunately this study did not include studies focussed at controlling *P. ficus* colonies living above ground. The difficulties faced with spraying EPNs onto the aerial parts are well known with regard to desiccation and ultra violet (UV) radiation being factors restricting the efficacy of using EPNs as a biocontrol agent. Despite this, effective adjuvants have been tested that reduce the negative effects of desiccation and UV. Considering the vertical movement of *P. ficus* colonies and the deciduous nature of grapevine, logic suggests that in the cooler months when the leaves have been shed, the colonies will be more grouped and 'concentrated' on the branches, cordons and trunk of the vine. This would be an opportune time to spray both the aerial parts of the grapevine, when there is a smaller surface area to the plant (having no leaves and grape bunches) and when the colonies are grouped together. The difficulty emerges when developing an un-biased method for sampling the mealybugs on the trunks and branches after the EPNs have been applied. The hidden behaviour, very small body size of *P. ficus* and the complex architecture of the grape vine trunk and branch surfaces lend themselves to be very difficult and challenging aspects of the study when considering the efficacy of aerial application. Hypothetically, if one were to spray EPNs to a vineyard infested with *P. ficus*, the researcher would need to sample as objectively as possible. To do this when a large part of the colony is not visible would be exceptionally challenging. Thus those sampled would be the visible individuals, when the individuals hiding under bark and deep within crevices are not sampled. These may be the individuals that are most highly impacted by the biocontrol. The micro habitats in such areas of the plant are also more conducive to the survival and insecticidal activity of the EPNs, due to higher humidity and protection from UV rays. Aerial semi-field trials could be an option to avoid such a bias, but in the instance of this study, pilot study attempts failed due to the movement, tiny size and inconspicuous behaviour of *P. ficus*. An unexplored option as an

aerial containment method will be to use cylindrical mesh cages filled with grapevine bark and Eppendorf tubes with mealybugs contained inside.

As a result this study needs to be extended to aerial applications, ensuring that the whole population (aerial and subterranean) of *P. ficus* has been tested and exposed to the applied EPNs. This will provide information describing the biocontrol agent's to full potential for controlling *P. ficus* in the field within an IPM scheme. The search for new EPN strains needs to continue in the hope to find those that are more effective at cooler temperatures and more resistant to harsh aerial conditions.